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JANUARY, 1933

CONTINUAL CORNIFIED VAGINAL CELLS AS AN  
INDEX OF AVITAMINOSIS-A IN RATS\*

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THE reliability of continual cornified vaginal cells in rats as a symptom of vitamin A deficiency has been the subject of several conflicting reports. Evans (3, 5), Macy, Outhouse, Long and Graham (11) and Hohlweg and Dohrn (7) consider the presence of cornified cells in the epithelial layers of the vagina to be the first and most delicate symptom of vitamin A exhaustion. On the other hand, Parkes and Drummond (14), and Coward *et al* (1, 2) conclude that continual cornified cells in the vaginas of rats are not a reliable index of A deficiency.

The material reported in this paper, collected in the course of experiments with rats on diets low in vitamin A, was studied to see, first, in what per cent of instances continual cornified cells in the vagina were associated with vitamin A depletion; secondly, to determine whether or not continual cornified vaginal cells were a more delicate gauge of the depletion of vitamin A than other symptoms; thirdly, to find whether there was any constant time-relation between daily cornified cells in the vagina and other symptoms of avitaminosis.

The stock from which most of the rats for this experiment were obtained came from animals reared by Helen Dean King at the Wistar Institute (10). They represent the 66th to the 68th generation of brother and sister mating. Of forty control animals, the modal age of the opening of the vagina was 36 to 39 days. The mean was 37 days with an S. D. of 3.30. Having the vaginas open at this time precluded any necessity of artificially rupturing the membrane, and allowed the smear to be watched soon after the rats were put on the experimental diet. One or more normal cycles could be observed before the effects of the diet became apparent.

\* The writer is indebted to the Department of Physiological Chemistry, Yale University School of Medicine, for advice and suggestions in carrying out this investigation.



The rats in Group IV, Tables II and III came from the Connecticut Agricultural Experiment Station.

The care and feeding of the stock animals from which the experimental rats were obtained were standardized as much as possible. Each pair of rats was kept in a separate cage. Strips of paper or cotton were used for bedding, which was changed once or twice a week. Trays, water bottles and tubes were washed and sterilized with like frequency. Stock animals were fed two types of diet. One group was fed 100 per cent calf meal, the other group was fed table scraps. Calf-meal is a commercial mixture containing approximately the following ingredients in 100 parts; oil meal 15, malted barley 10, whole wheat 22, oat flour 15, dried skim milk 15, yellow corn meal 20, ground limestone 1, steamed bone meal 1, salt 1. It is low in vitamin A. The table scraps include carrots, greens, cereals, milk, and meat, but no additional A is given in the form of cod liver oil.

Breeding females were mated at about one hundred days of age. All litters were reduced to six on the day of parturition.

Each experimental rat was isolated in a wire mesh cage. A wire base allowed feces to drop through. Partitions were kept between cages to prevent the spread of infection. The water bottles and tubes were washed and sterilized twice a week; cages were sterilized weekly. Rubber gloves were worn and were dipped in lysol after each animal had been handled. The animals were weighed twice a week. Once a week residual food was weighed and thrown out, the container sterilized and fresh food given. The weight of the spilled food was recorded in order that food-consumption might be determined. The animals were observed every day. If the food containers became empty at any time, supplementary food was given and recorded.

Vitamins B, D, and E were supplied by yeast, irradiation and hydrogenated vegetable fat.<sup>1</sup> Yeast<sup>2</sup> or irradiated ergosterol or a mixture of both was fed either four or six times a week in separate sterilized containers. One tenth of a gram daily of tested yeast was sufficient to produce a gain of weight in rats depleted of vitamin B. Two to eight tenths of a gram daily was fed to these experimental animals. Vitamin D was supplied by irradiation. The yeast, spread in thin layers, was exposed to the rays of a mercury vapor lamp at a distance of 15 inches for 15 minutes, then mixed, respread and irradiated for another 15 minutes (Hess 6).

Vitamin E was contained in the hydrogenated vegetable fat. Kennedy

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<sup>1</sup> Crisco.

<sup>2</sup> Tested yeast supplied by M. H. Givens of the Northwestern Yeast Co.

and Palmer (9) secured successful reproduction in rats on a purified diet containing 15 per cent of the fat, whereas replacement of the latter with 15 per cent of lard, resulted in sterility. Evans and Burr (4) secured "cures" in sterile rats, on E-free diets, by introducing 22 per cent of hydrogenated vegetable fat in the diet or by feeding daily supplements of 500 mg. of it during the gestation period.

The room temperature was adjusted between 80 and 84 degrees Fahrenheit. In this way it was possible to keep the rats alive for longer periods after severe symptoms of avitaminosis had developed.

Examination of the vaginal orifice was made every second or third day. After the vagina opened, smears were taken four to five times a week until the smears remained cornified for about ten days. After that, smears were taken five to six times a week on 16 animals, four times a week on 9 animals, and from two to three times a week on the remaining rats. The proportion and type of cells were recorded at each examination.

Smears were taken by the lavage method using tap water colored with neutral red. Small pipettes were made for this purpose. They were rinsed with mercuric chloride and then with water, or a fresh pipette was used for each animal in order to prevent infection.

A total of 101 female rats was used. Eight rats received Diets A or B shown in Table I. Ninety-three animals were fed diet No. 1 in Table I. Forty-one died or were killed before the vagina opened. All rats were killed immediately on the appearance of pneumonia. Records of the remaining 52 and the 8 rats on diets A and B are shown in Tables II and III.

The first group shown in Tables II and III was given extracted casein prepared according to the method given by Jackson (8). The second group was fed the technical casein without extraction. The third group, consisting of rats whose mothers were fed 100 per cent calf-meal diet, was given technical casein. The ingredients in the diet are in the proportions given in Table I.

All the rats in Groups I, II and III were weaned and placed upon the experimental diets at 21 to 24 days of age. At this time Groups I and II weighed approximately 39 grams and Group III weighed on the average ten grams less (Table III).

The average time in days at which the vaginas opened for the three groups (Table II) is 38.9 days (S.D.=7.1), 39.9 days (S.D.=6.2), 37.4 days (S.D.=6.9). The corresponding standard deviations of the means (S.D.M.) 1.9; 1.3; 1.8 show that the time of the opening of the vaginas in each group was essentially the same. The weight of the animals at this time

averaged over eighty grams for Groups I and II, Table III, (85.2 gm. S.D. = 12.5; 80.3 gm. S.D. = 10.9) and more than 20 grams less for Group III (56.7 gm. S.D. = 12.6).

The average time at which the rats show the continual cornified smear is about the same for the group wherein mothers were on table-scrap diet, with progeny on a diet including extracted casein, Table II, Group I, (43.4 days S.D. = 5.8) and for the group where parents were on calf-meal and progeny were fed technical casein (46.4 days S.D. = 4.6) Group III. Group II from mothers on a table-scrap diet fed technical casein showed continual cornified cells over two weeks later (60.9 days S.D. = 7.8). In Group I they probably started with a storage of the vitamin; in Group III the storage of vitamin was less, but the technical casein contained traces. The rats in Group II started with a storage of vitamin A and also had traces in their diet. This group had continual cornified cells considerably later. When the two groups bred from mothers on a table-scrap diet are compared (Groups I and II) the animals fed extracted casein show continual cornified cells over two weeks earlier than the rats fed technical casein. The weights are roughly in proportion to what might be expected

TABLE I  
PER CENT COMPOSITION OF VITAMIN A DEFICIENT DIETS

Ingredient	Diet No. 1.	Diet A	Diet B
Casein	15-18**	15	
Cornstarch	56-63**	22	
Hydrogenated vegetable fat*	18-22**		
Osborne and Mendel salt mixture IV	4	3	2
Lard		10	
Tobacco seed, ground		50	98
Yeast	0.4-0.8 gm. non-irradiated	0.2 gm. non-irradiated	0.2 gm. non-irradiated
Irradiated ergosterol	0.001-0.002 gm.	0.001 gm.	0.001 gm.

Note 1. The yeast and irradiated ergosterol were fed daily or every other day in a separate dish.

\* Crisco.

\*\* The rats ate more when the food was varied, so the diets were changed weekly or biweekly. The amounts of casein, cornstarch and Crisco were changed in the following proportions from 15, 63, 18, to 18, 56, 22 respectively.

Eighteen rats were weaned to a diet the same as above except that 0.8 gm. of yeast, half of which was irradiated, was given. In four instances no ergosterol was given until 6 to 9 days after weaning. In eleven instances no ergosterol was given until 21 to 28 days after weaning.

from the amount of stored and available vitamin A (Table III). Groups I and II average about one hundred grams (99.7 gm. S.D. = 10.4; 110.8 gm. S.D. = 15.1) and weigh on the average over twenty-four grams more than Group III (77.0 gm. S.D. = 14.6).

The failure to gain in weight or the appearance of xerophthalmia is considered pathognomonic of vitamin A deficiency. The average time for the appearance of xerophthalmia or these symptoms when weight-gain stopped shows a great difference in the three groups. Failure to gain in weight was recorded on the day that the weight did not show an increase over the last weight, and after which it remained the same or declined.

The first group of animals to show a loss of weight or xerophthalmia was composed of rats from mothers on a calf-meal diet fed technical casein, Group III, (58.7 days S.D. = 3.0). They weighed an average of 86.3 grams (S.D. = 17.1). The second group to show a loss of weight (Group I) consists of rats from mothers on a table-scrap diet fed extracted casein (70.3 days S.D. = 9.0) whose average weight was 123.8 grams (S.D. = 19.3). Last to show a second symptom of avitaminosis were the animals from mothers on a table-scrap diet fed technical casein (89.9 days S.D. = 14.0) whose weight averaged 126.3 grams (S.D. = 15.2). The animals to show the greatest amount of similarity within the group for the time in which xerophthalmia appeared or weight-gain stopped are those in Group III. Here the standard deviation of the mean is 0.8 as compared to approximately 3 for Groups I and II. This is to be expected because the parents were on controlled diets, low in vitamin A.

Seven rats died before the complete series of observations could be made; thus the number throughout the group is not constant. In four rats the vaginas did not open in the first forty days. In each instance when the vagina opened cornified cells were present and continued cornified. There was no way to tell how long they had been present; hence these animals could not be used in computing the interval in Table II. The interval was estimated on 48 animals by subtracting the age of the animal at the appearance of continual cornified cells in the vaginal smear, from the age of the animal when the second symptom of avitaminosis appeared or, as in Group IV, the time the experiment was terminated. These individual differences were averaged and the dispersion-measures computed directly.

The interval between the appearance of cornified cells in the vagina and the second symptom of avitaminosis is about the same for Groups I and II, Table II, (27.8 days S.D. = 9.0; 28.3 days S.D. = 15.0). In Group I, however, both symptoms appear at an earlier age than in Group II. The

interval between the first and second symptom of avitaminosis in Group III (12.5 days S.D. = 4.8) of animals from mothers on a calf-meal diet is less than half that of Group I or II of animals from mothers on table-scrap diets. The more rapidly the animal is depleted of vitamin A, the shorter the interval. It is interesting that even in this group the appearance of

TABLE II  
AGE OF RATS ON VITAMIN A-DEFICIENT DIET  
(age in days)

Vagina opened	Continued cornified smear	Appearance of xerophthalmia or weight gain stopped	Experiment terminated	Interval*
Group I:** rats on A-deficient diets made with extracted casein; mothers on table scrap diet.				
Number of rats 14	10	12		10
Mean 38.9	43.4	70.3		27.8 Significant
S.D. 7.1	5.8	9.0		9.0
S.D.M. 1.9	1.8	2.6		2.8
Group II:** rats on A-deficient diets made with technical casein; mothers on table scrap diets				
Number of rats 24	20	19		19
Mean 39.9	60.9	89.9		28.3 Significant
S.D. 6.2	7.8	14.0		15.0
S.D.M. 1.3	1.7	3.2		3.4
Group III:** rats on A-deficient diets made with technical casein; mothers on 100 per cent calf meal.				
Number of rats 14	12	14		12
Mean 37.4	46.4	58.7		12.5 Significant
S.D. 6.9	4.6	3.0		4.8
S.D.M. 1.8	1.3	0.8		1.4
Group IV:*** rats on A-deficient diets made with tobacco seed.				
Number of rats 8	7	—	8	7
Mean 76.0	111.3		169.4	69.9 Significant
S.D. 28.3	29.9		38.6	25.7
S.D.M. 10.0	11.3		13.7	9.7

\* Interval represents the difference between the appearance of continued cornified smears and the appearance of xerophthalmia, or weight gain stopped, or as in Group IV the time the experiment was terminated, of each rat subtracted separately then averaged.

\*\* The rats were weaned and placed on the experimental diets at 21 to 24 days of age.

\*\*\* The rats were placed on the experimental diets at from 28 to 37 days of age.

continual cornified cells precedes other symptoms. It is conceivable that in rapidly depleted groups the two symptoms might appear simultaneously.

In order to see whether a group of animals could be maintained with a certain amount of vitamin A, such that cells of the vagina would become constantly cornified without the appearance of any other symptom of avitaminosis, the following experiment was done. Eight rats, divided into two groups of four each, were put on Diets A and B from 28 to 37 days of age (Table I). Their history is given in the fourth group of Tables II and III. The vaginas opened late compared to the inbred rats from the King stock which have an early maturity (mean 76.0 days S.D. = 28.3). Their weight at this time averaged 104.9 grams (S.D. = 18.3). Continual cornified cells did not appear until about 111.3 days (S.D. = 29.9) because the food contained some vitamin A. The weight averaged 141.7 grams (S.D. = 30.0). No other symptoms developed. When the animals weighed 150 grams, or over, there was some fluctuation in their daily weight. Unless an infection developed, there was no pronounced continuous drop in weight. In one instance the cells remained cornified for 126 days. Cornified cells might have remained for the entire normal life of the animal if the experiment had not been terminated in each instance by administering cod liver oil or by death. From twenty-four hours to three days after the dose of cod liver oil, continual cornified cells disappeared and the normal cycles were observed. Group IV cannot be compared directly with the other three groups. The rats did not come from the same parents and were not fed similar diets.

In the first three groups, however, the difference in time of the appearance of cornified cells and the second symptom of avitaminosis is statistically significant. In Group IV, by design, the animals were allowed to continue under fixed dietary conditions for an interval greatly in excess of that required for the other three groups. Since a second symptom of avitaminosis did not develop, the intervals between the appearance of cornified cells and the termination of the experiment were calculated and found to be statistically significant.

The first indication of a change in the oestrus cycle appeared as a lengthening of the oestrus and a shortening of the dioestrous interval. Gradually the cornified phase extended until it had become continuous. About the time the cells became cornified, the vagina seemed very dry (Manville, 13). The animals had to be handled carefully in order to prevent trauma to the mucosae of the vagina when taking the lavage. In spite of every pre-

caution some animals had necrosis and sloughing of the mucosae. This was shown in the smears by an excessive number of leucocytes, the presence of discrete red blood cells, or by hemorrhage.

In every rat where the smear had shown cornified cells for two weeks or longer, leucocytes in the vagina were reported at some time in its history.

TABLE III  
WEIGHT OF RATS ON VITAMIN A-DEFICIENT DIET  
(weight in grams)

Put on diet	Vagina opened	Continued cornified smear	Appearance of xerophthalmia or weight gain stopped
Group I: rats on A-deficient diets made with extracted casein; mothers on table scrap diet.			
Number of rats 14	14	10	12
Mean 39.3	85.2	99.7	123.8
S.D. 5.1	12.5	10.4	19.3
S.D.M. 1.4	3.3	3.3	5.6
Group II: rats on A-deficient diets made with technical casein; mothers on table scrap diet.			
Number of rats 24	24	20	19
Mean 37.8	80.3	110.8	126.3
S.D. 7.2	10.9	15.1	15.2
S.D.M. 1.5	2.2	3.4	3.5
Group III: rats on A-deficient diets made with technical casein; mothers on 100 per cent calf meal.			
Number of rats 14	14	12	14
Mean 28.3	56.7	77.0	86.3
S.D. 4.0	12.6	14.6	17.1
S.D.M. 1.0	3.4	4.2	4.6
Group IV: rats on A-deficient diets made with tobacco seed.			
Number of rats	8	7	
Mean	104.9	141.7	
S.D.	18.3	30.0	
S.D.M.	6.4	11.3	

The time when leucocytes are present is significant. In some rats on A-deficient diets they were present in only one smear during the period of constant cornification. In others they might be mingled with the cornified cells for weeks at a time. This is quite different from their rhythmic appearance in the normal animal. Out of a total of 506 smears taken after

the vagina had been constantly cornified, 119 (24 per cent) contained leucocytes.

The vaginas of 33 rats were sectioned for further study. In every instance cornified cells were seen at the periphery. Five rats showed necrosis of the mucosae. One had a recent hemorrhage with free blood in the lumen. Some of the animals had been on the A-deficient diet a long time before being killed, which would probably make the incidence of infection higher. In 17 out of 28 specimens of intact mucosae, leucocytes could be seen migrating between the cornified cells. The changes in the vagina are similar to the keratinization found by Wolbach and Howe (15) for other epithelial tissues of the body.

Mason finds that testicular damage occurs in the absence of vitamin A (12). He has observed considerable testicular damage, especially in chronic deficiency of vitamin A of a mild type, without xerophthalmia (personal communication). In the female rat, similarly, vaginal changes precede other symptoms of avitaminosis of A.

There is a definite sequence with which various structures of the body are affected by an inadequate amount of vitamin A. It may be that the vagina becomes affected first because it represents unusually active epithelium. Or again, the cells of all of the affected membranes may become keratinized at about the same time. Cornification may appear earlier in those membranes most exposed to drying, as the vagina and cornea, but xerophthalmia may not be apparent until the cornified cells of the eyelids have become the seat of infection.

#### CONCLUSION

Continual cornified vaginal cells appeared in 100 per cent of the rats on diets deficient in vitamin A, where the animals lived to show avitaminosis. Histological section of the vaginas of 33 rats showed the peripheral cells of the vagina to be cornified. Cornification of the vagina invariably preceded other symptoms of avitaminosis; the time elapsing was positively correlated with the amount of vitamin contained in the diet or stored in the animal. In this group of 60 rats cornified cells in the vagina were the most delicate indication of avitaminosis of A.

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# STANDARDS FOR PREDICTING BASAL METABOLISM

## I. PREDICTION FOR GIRLS FROM 17 TO 21

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THE adequate defining of physiological normals in the field of basal metabolism has been of necessity a slow process because of the multitude of factors which can modify the findings, many of them subtle and difficult to evaluate. Both the greater difficulty of standardizing experimental conditions with immature subjects, and the greater physiological variability of the growing periods have retarded knowledge of the exact configuration of the age-curve of metabolism in childhood and youth. There are several interesting details of this curve which await clearer definition by the compiling of statistically significant volumes of normal data. Among these is the period around the age of puberty. Another is the point where the slope of the curve flattens out as the adult type of metabolism becomes stabilized. It is with the latter part of the curve for women that the present study is concerned.

The study was undertaken in the effort to determine the reference standards most suitable for practical use in connection with student health at the University of Wisconsin. In such an institution young women come in for a large share of the metabolic studies that are carried on. Interpretation of results has been greatly hampered by the serious inconsistencies of available predictions for such subjects. These inconsistencies can be traced both to relative paucity of data for ages between 17 and 21 at the time when the classical reference standards were being formulated, and to wide differences in methods that were resorted to for temporarily bridging the gap.

The three predictions from authoritative sources which are most widely referred to in America have here been considered: the lower ranges of the original Aub-DuBois tables (1); the special prediction for girls from 12 through 20 proposed by Benedict in 1924 (4); and the Harris-Benedict prediction for adult women (15), after extrapolating for ages under 21. In endeavoring to compare these it becomes evident that not only do wide discrepancies in average absolute level exist between their predictions,

but the values of the Benedict prediction, built up by methods of study very different from any other standard in the field, do not run parallel to those of either of the others for girls of different body builds. Thus a girl of 17, 157 cm. tall and weighing 47.3 kg., whose measured heat-production is found to be 1166 calories for 24 hours would be reported as showing a metabolic rate of  $-16$  by DuBois,  $-12$  by Harris-Benedict, and  $+13$  by the Benedict prediction; whereas a girl of 18 years, 157 cm., and 66.4 kg., with heat-production determined experimentally as 1387 calories per 24 hours would show rates within the "normal" range by all three predictions, i.e.,  $-9$  by DuBois,  $-7$  by Harris-Benedict, and  $-4$  by Benedict.

Being unable to make a decision between these on purely theoretical grounds, we set out to collect a sufficiently large number of data on normal girls under 21 to make possible either a convincing choice between the older standards, to indicate how one of these could be modified to meet our needs, or, if necessary, to serve as basis for formulating a new standard of reference in clinical cases from the same source as the normals studied. This last was made possible through the coöperation of the Department of Mathematics of the University, after data were on hand for 97 girls of ages quite uniformly distributed between the years 17, 18, 19 and 20. This standard has been included with the three older ones in various comparisons on our own test data and finally on a comparably large series of normal controls in the same age range reported in several recent studies from widely different localities.

The results of these various studies and comparisons have encouraged us to believe that our data are of more than local significance. In offering the prediction standard we do so primarily for its immediate usefulness and for the interest of the comparisons upon which it was based and which it may suggest. The gain in prediction accuracy which it offers over the best of the older standards, after correcting the latter for differences in mean absolute level, is slight, though consistent for both the Wisconsin and other data considered. The comparisons demonstrate the logic of considering girls of college age along with adult women in attempting to predict their metabolism.

It is generally conceded that the standards for adult women are too high (22, 5, 13), and this has been found particularly true in our hands for younger women. But permanent recommendations for correcting the older standards can be justified only after many more data have been compared over the largest possible range of ages. There is no assurance that any single recommendation for correction of mean values of any of the stand-

ards will be valid for all age-ranges or even for different groups of controls of the same ages and differing in average bodily configuration. The present comparative study is offered for whatever bearing it may have in the eventual reaching of some general agreement on the subject of standards for women.

### EXPERIMENTAL

The rather large bulk of fundamental data connected with the study has been put on record in the *Transactions of the Wisconsin Academy for Sciences, Arts and Letters* for 1932 for the interest of those who may care to make more detailed examination of the individual measurements or to use any parts of the data for comparisons.

*The subjects* were students who entered the University with a grading of A in their medical and physical examinations and whose histories indicated good health since coming to school. Cervical inspection and supplementary questioning of each subject at the time of her test were designed particularly to rule out suspicion of thyroid disease or history of significant menstrual irregularity or difficulty other than common degrees of discomfort. Experience showed it to be undesirable to attempt to get satisfactory results at certain times of the school year, such as periods soon before or after vacations or examinations, prominent social events, or athletic exhibitions in which subjects who came from the Physical Education Department were participating. This conclusion was reached after it became apparent that rates obtained at such times were apt to be distinctly high in comparison with later data on the same subjects, while pulse-rates and erratic respiration often pointed to inadequate relaxation.

Our aim was for essentially uniform distribution of ages. In the data submitted to the final analysis there were represented 25 subjects each of the ages 17, 18 and 20; and 22 of age 19, including in each classification all those within 6 months of the designated year. A total of 163 acceptable tests was obtained on the 97 subjects. Intervals between tests were 3 weeks in 35, and 7 to 55 days in the remainder. First accepted tests only on each subject were used for the prediction standard, for reasons that will be mentioned later. The other tests were used for making various comparisons.

*Geographic source of subjects.*—Eighty per cent of the students were drafted from newly entering classes, so that their prevailing living conditions can with fair safety be considered as determined by their homes. The mid-west of course predominated largely, furnishing 85 per cent of the

subjects, but 17 states in all were represented. Roughly ten times as many of the subjects were from goiter-belt as from non-goiter-belt regions (23), so that no formal comparisons were justified. It was of passing interest, however, to find that plus and minus metabolic rates (Wisconsin standard) were similarly distributed in the two groups.

*College classification.* One-third of the subjects were registered in the Physical Education course at the University. This relative preponderance may be attributed to several factors: the physical fitness of these girls, their early registration, and their willingness to cooperate. When the metabolic rates of these subjects were tabulated separately from those of the rest of the group, no distinctive tendencies to high, low, or unusually variable rates could be detected. Any actual tendency to greater variability in heat-production, sometimes said to obtain for the physically active, in distinction to relatively more sedentary types of individuals, could perhaps be offset in this particular group as a whole by the stabilizing effect of more perfect relaxation as the result both of physical discipline and ready understanding of the aims of such an experiment.

*Thyroid observations.* The criteria of what constitutes a strictly "normal" thyroid gland must vary somewhat according to the region in which the observations are made. Someone has observed that some degree of enlargement of the gland is so common in young women in our middle states that even those illustrated on magazine covers are apt to show slight evidence of goiter! Certainly it would have been impossible for us to assemble any significant sized group for study if we had discriminated against simple enlargement of the gland. Hence, as long as all our other criteria of normal health were satisfied—which excluded carefully, of course, all suspicion of *symptoms* referable to the thyroid—we accepted the girls as they came to us. When the results of the special examinations made at the time of the tests were tabulated it was found that only 2 of the 97 girls were recorded as having strictly negative thyroids—i.e., neither isthmus nor lobes found visible or palpable. On the other hand, there were 24 (26 per cent) in whom enlargement was definite enough to be considered in the author's judgment as mild or moderate degrees of simple goiter. All of the latter were from regions classed as goiter-belt or border-line. They did not show any definite trend in metabolic rates, as a group.

Most of the subjects from goiter-belt regions have histories of having used iodine in some form as goiter prophylaxis, usually for short periods only. If such medication serves its admitted purpose—i.e., of replacing an element deficient in the natural ration, it should presumably tend to make

this group if anything more comparable, than otherwise, with those from regions where the necessary iodine is obtained in the food.

*Menstruation.* After consultation with members of the clinical staff, it was decided to eliminate results on any subjects who gave history of irregularity in periods of a week or more, or of dysmenorrhea sufficient to be admitted as incapacitating for any time. The data on subjects whose tests were satisfactory but who had to be discarded as not strictly normal by these requirements have been set aside for future study, since one of the problems in which we are specifically interested is the connection between basal metabolic rate and menstrual history in women not definitely in ill health.

Catamenia without discomfort at the time of a test we did not allow to disqualify the test. The state of the literature on this subject at the time our investigation was undertaken did not suggest that this discrimination would be necessary or even desirable in a study of this sort. As the study progressed, the thoroughgoing investigations published by Benedict and Finn in 1928 (6), by Hitchcock and Wardwell (16) and by Griffith *et al.* in 1929 (14), and by Conklin and McClendon in 1930 (9), served to crystallize the general impression derived from earlier reported work, namely that cyclic changes in the heat-production of women can be demonstrated by adequate methods of study and analysis. The very difficulties that have beset the final demonstration of this periodicity point to the fact that the cyclic changes are of a relatively minor order of magnitude, and that the maxima and minima vary greatly in their position in the cycle from one woman to another, and even in different cycles in the same woman. Apparently the changes in metabolic rate that can be correlated with the menstrual cycle consist of a low phase either during or shortly following the period, with a high one of about the same order of magnitude recurring some time intermenstrually. The position of the intermenstrual rise has been variously placed as about mid-way between the periods, or shortly before the beginning of a period—i.e., pre-menstrually. Since there are both maxima and minima of about the same relative extent, it would hardly seem more logical to try to avoid the menstrual (or post-menstrual) low points than the intermenstrual high ones in a study that aims to define reasonable ranges of variation of the basal metabolism of normal women for purposes of prediction. If one avoided both types of changes, there would be left only a short intermenstrual interval for making the tests, and there would still be decided uncertainty as to just which interval this would be.

It seems desirable, however, to record the phase of the menstrual cycle at which tests are made in women. This we have done in the majority of the present tests. Perhaps future studies will confirm the suspicion voiced by Lanz in 1925 (21) that though such cyclic changes are apparently of minor importance in normal women, they may perhaps become exaggerated not infrequently in those who are ill, particularly with some of the endocrine disorders.

*Technic.* The tests were made in the metabolism laboratory of the Wisconsin General Hospital under the same general conditions used for the study of out-patients, but in the interest of unity and control in the series, were handled with very rare exceptions by the same operator. Appointments were made between 7:30 and 8:30 a.m. Each student who was given an appointment, was furnished with the instructions for preparation and brief description of the routine which the hospital provides for patients who come in for the test. The usual requirements for preparation were followed—i.e., the subjects came without breakfast, and were required to lie quietly with enough covering to be comfortable, for at least 30 minutes before the test.

A closed circuit type of apparatus was used—in an occasional test at the beginning of the series the “Sanborn Grafic” was used, but for all the rest, the Benedict “Portable” model with motor-driven circulation. The machines were kept at all times free from leaks or other determinable defects. During each run a confirmatory leak-test was made by Benedict’s expedient of placing a weight on the spirometer midway through the run and noting the point in the tracing where the weight was applied.

At least two technically acceptable runs were required for each test. The lowest run was accepted as determining the rate for any given test, but all accepted runs were figured separately for the information to be gained by their comparison. This is our routine practice, since with the technic used we feel that any error which might conceivably arise without being detected would tend to raise, rather than lower the readings.

All tests were accepted for analysis unless specific grounds for their rejection were apparent in technical flaw, lack of coöperation or indisposition of the subject at the time of the test. One of the things we were interested in testing out was whether ranges of variation similar to those considered reasonable in work with adults would be found in these measurements of younger subjects.

*Measurements taken* were defined as follows: the body *weights* recorded were nude weights. *Age* was taken to the nearest birthday. In view of the

emphasis that has been placed by many observers upon the value of the *sitting height* for making physiological comparisons, this measurement was taken for these subjects in addition to the *standing height*, in case the former should prove to furnish the more accurate factor for prediction of the metabolism. *Calories* of "basal" heat-production were calculated from the oxygen consumption as measured during the lowest run within any given test, by the usual assumption of the average post-absorptive respiratory quotient for which the caloric equivalent of oxygen is 4.825 per liter (8). In calculating calories per square meter, *surface area* has been estimated by the height-weight chart of DuBois and DuBois (11). *Metabolic rates* have been figured in each test according to 4 predictions including our own, which will be discussed later.

## RESULTS

### *I. General Comparisons Among Wisconsin Test Data*

*The agreement between duplicate runs within tests* was figured in 170 tests on the 97 subjects of the standard series, plus 7 examined after the data for the standard were under analysis. Percentage differences were figured on the basis of the lowest run in each case. Twenty-six tests showed no difference between successive runs. Of the rest, 82 showed lowest readings in the first runs, and 62 in runs after the first. In 72 per cent of the total number the discrepancies were within 3 per cent of the *low run*; in 88 per cent within 5 per cent; and in all, within 10 per cent. Our impression gained from general experience with untrained adult subjects is here confirmed—namely, that duplicate runs in the large majority of well-controlled tests can be expected to agree within 5 per cent of the lower run, while there will always be some larger differences that are not definitely accounted for.

*Agreement between repeated tests on the same individuals.* Of the 70 instances in which more than one acceptable test per subject was obtained, 30 showed lower figures for oxygen consumption in the first tests, and 40 in later ones. The magnitude of the discrepancies was in this case calculated on the basis of the first accepted test for the individual—that is, the figures show changes in measured heat production after the first tests. In 47 per cent of the cases these changes were within 3 per cent of the first results; in 64 per cent within 5 per cent; and in 93 per cent within 10 per cent.

*Pulse-rates* were counted routinely twice during each run for supplementary evidence as to the success with which relaxation was maintained. The following average rates observed in the different age-groups of the Wisconsin series suggest that on the whole relaxation was maintained



almost as well in first as in later tests. In individual cases of course there was much variation in both directions from the picture for the group, since the pulse-rate and metabolic rate, as is well known, vary only roughly parallel with each other.

TABLE I  
AVERAGE PULSE-RATES—WISCONSIN STD. SERIES.

Age	All first tests:		Subjects accepted more than once	
	Aver.	Range	Aver. first tests	Aver. later tests
17	69	48-86	70	68
18	69	53-86	69	69
19	68	43-89	71	63
20	72	59-88	71	69
All:	70	43-89	70	68

It is interesting to note that the minimum which we found in three of these four groups of healthy American girls is lower than the minimum (54 p.m.) found by MacLeod, Crofts, and Benedict (22) in their study of 9 Oriental young women who had lived in this country for from 15 months to 4.5 years. The average tendency for what we consider low levels of such vital functions as pulse and metabolism in Orientals, even after prolonged exposure to our conditions of living, is probably considered most logically as an indication of a constitutionally superior capacity for repose on the part of these races, rather than an essential difference in metabolic processes. It would appear, then, that a good many of these girls were quite comparable to the Orientals in this respect.

*Body build.*—Figure 1 gives a picture of the height-weight relationships in the 97 Wisconsin standard subjects. The heavy line shows the average for the group, given by the regression line of weight on height, and the broken lines mark off distances on either side equal to the standard deviation for weight. We were interested to find that the regression equation predicted almost the same weights for height as Bardeen's tables (2), which we use for clinical standards. The Wisconsin group is evidently anthropometrically representative.

*Menstruation and Metabolic Rates.* Of the 97 first tests used for the Wisconsin Standard, 69 have been placed as to time since the beginning of the last menstrual period. Probably few of the unplaced ones were on menstrual days, since the girls who came during the catamenia usually volun-

teered that information. Considering all tests on the standard and extra subjects examined by the author, information is at hand for identifying time in the cycle of 114 tests. The cycle was divided into 5-day periods, including the first 5 as menstrual days, and average metabolic rates according to the Wisconsin standard were computed for each interval.

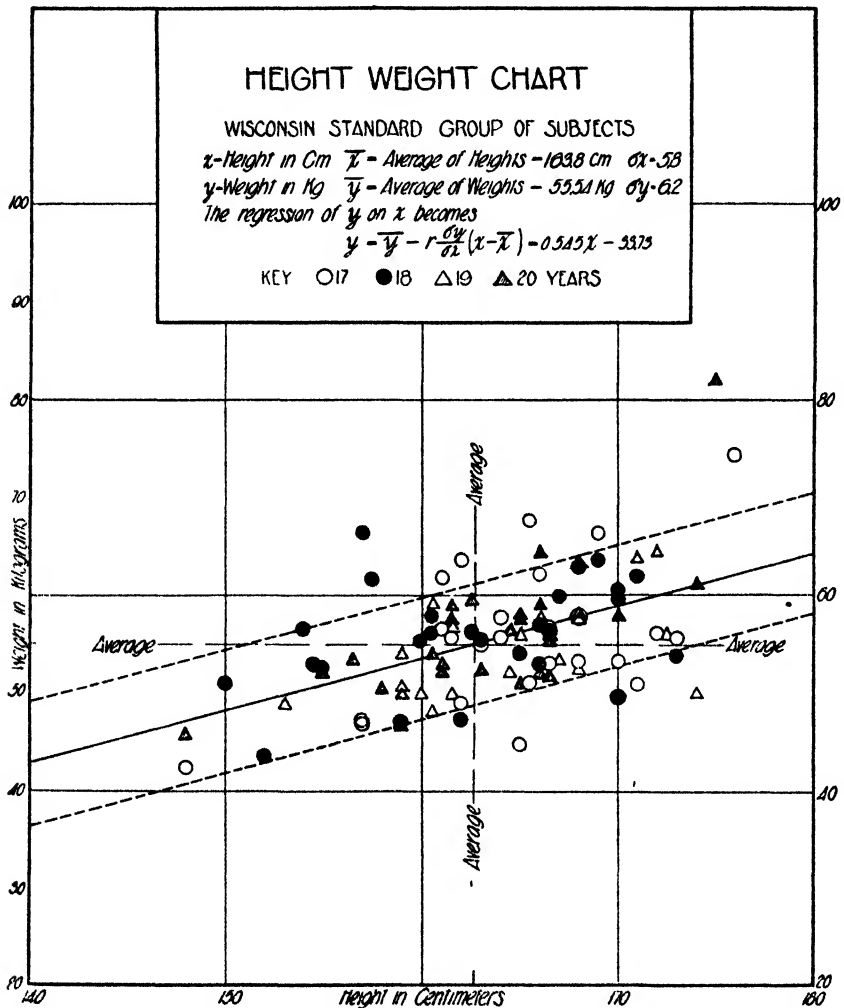


FIG. 1

Though little information was anticipated from the averaging of such scattered tests, there nevertheless was indicated a tendency to values somewhat lower than the intermenstrual average during or immediately follow-

ing the period. The exact time of an intermenstrual high point, also indicated here, varies according to the grouping of days chosen for averaging. The following values were obtained:

For the 69 first tests: the average rate for 5 menstrual days was  $-2.20$  per cent, and those for successive 5-day intervals thereafter were  $-2.70$ ,  $+2.07$ ,  $+0.075$ ,  $+1.18$ , and  $\pm 0$ . Intermenstrual average:  $+0.12$ . There were 10 menstrual tests and 10, 14, 16, 11 and 8 in succeeding 5-day periods.

For all 114 classified tests the average rate for 5 menstrual days was  $-2.21$  per cent, and those for successive 5-day intervals thereafter were  $-2.13$ ,  $+1.96$ ,  $+1.48$ ,  $-1.06$ , and  $-1.15$ . Intermenstrual average:  $-0.07$ . There were 14 menstrual tests and 24, 24, 21, 18 and 13 in succeeding 5-day periods.

The average differences are thus small, as would be inevitable from the large effect of both inter- and intra-individual variabilities in such a series, but the average menstrual lowering of the rate is consistent in the two series. The two lowest individual rates did not occur during the menstrual period in these series, but the two highest were intermenstrual. Of 14 menstrual rates in the larger series, 10 are below the average Wisconsin prediction, which happens to coincide almost exactly with the intermenstrual average of those tests whose position in the cycle could be placed. Evidently among those which could not be so placed by the records at hand there were just enough rates above the Wisconsin prediction to compensate for the lower menstrual averages among those tests analysed.

## *II. Statistical Analyses, and the Wisconsin Prediction Standard*

We decided to use *first accepted tests only*, for the prediction standard, so only these were submitted to the more formal mathematical analyses. The others were reserved for comparisons. This course was adopted after considering the two more obvious alternatives: 1.—that of averaging all available tests for each subject; or 2.—that of using the lowest test obtained in each case as perhaps representing more nearly true “basal” conditions. The former would have smoothed out some of the spread in the data, and the latter (and to a less extent also the former) would of course have lowered our base-line, neither of which we have considered necessarily desirable from the point of view of prediction. Either, to be strictly applied, would have required our having more than one acceptable test on every individual in the series, which, however, we had not set as our goal. The idea that underlay our decision was that we were interested primarily in

arriving at some concrete ideas, not of ideal rates of heat-production, and not only of their average, probable level, but rather in the whole *range* of variability within which individual determinations might reasonably be expected to fall for subjects of these ages not especially trained, but studied under well-controlled standard "basal" conditions.

After some rather extensive preliminary analyses, the method of multiple correlation, making metabolism a linear function of the height and weight, was decided upon as most suitable for the data at hand.<sup>1</sup> Average measurements and the most important of the statistical constants calculated for the 97 sets of measurements considered by separate and combined age-groups may be found elsewhere (25).

For the first two groups that were completed for analysis, equations were formulated in which measurements for sitting height were incorporated as well as, or instead of, standing height measurements. Comparisons, including those of the standard errors of estimate by the different types of equation, made it evident that the use of the sitting height did not add anything of value from the point of view of accuracy in predicting. Therefore the less usual and probably less accurate measurement was dropped.

While 25 cases for each year proved to be ample for defining the influence of height and weight on the metabolism with a good degree of practical certainty, a much larger number would have been necessary to determine an age-factor within the narrow range of ages of the present study. The changes in heat-production with each year of age over this period are evidently so small as to be readily obscured by incidental fluctuations. We shall hope to incorporate the benefit of such a factor when our observations shall have been extended to cover a larger range of years. Meanwhile, for practical purposes of prediction, the age-factor is small enough to be safely neglected within this range.

In a general way these data show an unmistakable downward trend of heat-production from the younger to the older girls. This is brought out most consistently when the 17 and 18, and the 19 and 20 year groups are combined to smooth out some of the individual variations. This has been done in Table II for the data of all tests of our series. For logical inter-individual comparison, heat-production is expressed in the final column in calories per unit of body surface.

One of the less obvious pitfalls in trying to define small differences in

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<sup>1</sup> Curves obtained by expressing the logarithm of the metabolism as a linear function of the logarithms of the height and weight proved to be sensibly the same as those based on the simple measurements for the ranges of values dealt with.

measured heat-production from one group to another is suggested when we examine the averages of first and later tests separately. Inspection of Table II reveals a considerably larger drop between first and repeated tests in the younger than the older girls. It appears probable that some part of the observed difference in average levels of heat-production, therefore, is apparent, and due to better relaxation on the part of the older girls as a group.

TABLE II  
AVERAGE HEAT-PRODUCTION OF GIRLS 17-18 AND 19-20 YEARS OLD  
All tests Wisconsin Standard Series

Subjects		Ages	No. of subjects in group	No. of tests in group	Average calories/24 hrs.	Average cal./Sq. M./hour
All Tests, Subjects who contrib. to U. W. Std.		17-18	50	89	1313	34.40
		19-20	47	74	1280	33.54
Those who had more than one test	First tests	17-18	39	39	1330	35.00
		19-20	25	25	1284	33.57
	Later tests	17-18	39	39	1304	34.14
		19-20	25	27	1272	33.43

Our final decision was to incorporate the combined data for the 4 years from 17 through 20 into the simple prediction standard given as Table III. The prediction is based upon standard methods of multiple correlation and represents the equation:

Basal heat production in calories for 24 hours =  $10.63 \times \text{weight in kg.} + 3.23 \times \text{height in cm.} + 184.61$ .

### *III. Comparisons of Suitability of Various Standards for Predicting the Heat Production of Girls from 17-21.*

A composite set of data on 119 normal controls has been collected for concrete comparisons as to the suitability of different prediction standards for girls of the ages we have studied. In order to come to any decision as to the suitability of our own prediction, which we preferred to use until there could be some more general agreement on the manner and extent to which existing standards must be modified to meet more modern conditions, of course it was necessary to try it on at least a comparably large number of data other than those from which it was constructed. We began by assembling what records we could on other normal controls of the same ages

TABLE III  
 PREDICTION TABLE FOR BASAL METABOLISM OF YOUNG WOMEN—AGES 17-21  
 University of Wisconsin, 1930  
 Height in Centimeters

	140	145	150	155	160	165	170	175	180
40	1062	1078	1094	1110	1127	1143	1159	1175	1191
41	1073	1089	1105	1121	1137	1153	1170	1186	1202
42	1083	1099	1116	1132	1148	1164	1180	1196	1212
43	1094	1110	1126	1142	1158	1175	1191	1207	1223
44	1105	1121	1137	1153	1169	1185	1201	1218	1234
45	1115	1131	1147	1164	1180	1196	1212	1228	1244
46	1126	1142	1158	1174	1190	1207	1223	1239	1255
47	1136	1153	1169	1185	1201	1217	1233	1249	1266
48	1147	1163	1179	1196	1212	1228	1244	1260	1276
49	1158	1174	1190	1206	1222	1238	1255	1271	1287
50	1168	1184	1201	1217	1233	1249	1265	1281	1298
51	1179	1195	1211	1227	1244	1260	1276	1292	1308
52	1190	1206	1222	1238	1254	1270	1286	1303	1319
53	1200	1216	1232	1249	1265	1281	1297	1313	1329
54	1211	1227	1243	1259	1275	1292	1308	1324	1340
55	1221	1238	1254	1270	1286	1302	1318	1335	1351
56	1232	1248	1264	1281	1297	1313	1329	1345	1361
57	1243	1259	1275	1291	1307	1323	1340	1356	1372
58	1253	1270	1286	1302	1318	1334	1350	1366	1383
59	1264	1280	1296	1312	1329	1345	1361	1377	1393
60	1275	1291	1307	1323	1339	1355	1372	1388	1404
61	1285	1301	1318	1334	1350	1366	1382	1393	1414
62	1296	1312	1328	1344	1360	1377	1393	1409	1425
63	1306	1323	1339	1355	1371	1387	1403	1420	1436
64	1317	1333	1349	1366	1382	1398	1414	1430	1446
65	1328	1344	1360	1376	1392	1409	1425	1441	1457
66	1338	1355	1371	1387	1403	1419	1435	1451	1468
67	1349	1365	1381	1397	1414	1430	1446	1462	1478
68	1360	1376	1392	1408	1424	1440	1457	1473	1489
69	1370	1386	1403	1419	1435	1451	1567	1483	1499
70	1381	1397	1413	1429	1446	1462	1478	1494	1510
71	1392	1408	1424	1440	1456	1472	1488	1505	1521
72	1402	1418	1434	1451	1467	1483	1499	1515	1531
73	1413	1429	1445	1461	1477	1494	1510	1526	1542
74	1423	1440	1456	1472	1488	1504	1520	1536	1553
75	1434	1450	1466	1483	1499	1515	1531	1547	1563
76	1445	1461	1477	1493	1509	1525	1542	1558	1574
77	1455	1471	1488	1504	1520	1536	1552	1568	1585
78	1466	1482	1498	1514	1531	1547	1562	1579	1595
79	1477	1493	1509	1525	1541	1557	1573	1590	1606
80	1487	1503	1520	1536	1552	1568	1584	1600	1616

at this university. Besides the 7 subjects studied after the data for the standard were under analysis, there chanced to be available measurements made in connection with two graduate theses. These dealt with a group of 21 presumably normal sorority girls studied by Jean Fish in 1928 for a thesis in psychology; and a series of 9 presumably normal students investigated by W. C. Shipley in 1930, also in connection with a problem in psychology. These together constitute a supplementary Wisconsin series of 37 cases. The two psychology studies were made independent of the hospital under carefully controlled experimental conditions, for the physiological comparisons which they might afford.

For a more crucial test we have used two large series of controls which came to our attention in 1930 and 1931 in reports of the metabolism of young women from non-goiter-belt regions. These are the study of the basal metabolism of young college women in Florida by Jennie Tilt (26), and a study by Remington and Culp (24) of the basal metabolic rates of student nurses at Charleston, South Carolina. Individual measurements, rather than merely group averages, were fortunately available in these reports.

Twenty-nine of Tilt's subjects were between 17 and 20.5 years, and hence comparable with our group. The technic used was similar to ours and Miss Tilt calculated the rates of her subjects by both the Harris-Benedict and the Aub-DuBois predictions. By both standards the rates of most of the subjects were decidedly low, and the author concluded that the basal metabolism of young women in Florida tends to be significantly lower than that predicted for young women of the same ages and living in the north.

Of the 93 student nurses examined by Remington and Culp, 48 were within our required age-range, and thus afforded another large homogeneous group for comparison. The technic again was similar in principle to ours. These authors also noted the prevalence of low rates according to the DuBois standard, but having observed that both lower and higher figures have been reported from studies made in northern states, concluded that the low values could not be regional. They suggest nutritional level as a possible factor in trend of metabolic rates, though they feel that this factor must be slight in groups of normal individuals. They did not consider the possible bearing which the choice of standards might have on warranted interpretations.

We were interested to see how our data compared with the more modern studies of normals from the Nutrition Laboratory of Boston, but found just 5 subjects within the range of our study in Benedict's 1928 series of

controls (5). Benedict made a definite effort in this later series to secure a wider range of body builds in his subjects than had been the case in the original Nutrition Laboratory series, since this should make possible better definition of the relationships between metabolism and body measurements when the latter are far from average. Three of these five girls are as it chances, heavy in proportion to their heights. As a separate group the small series is therefore unbalanced; but for this very reason, as well as its source, it has proved to offer some most interesting comparisons.

The fundamental data on the 119 subjects of the above composite series, together with notations of the recalculations and derived data which we have added for comparison are on record in the Transactions of the Wisconsin Academy (25).

*Prediction standards compared.* Metabolic rates have been calculated as percentage deviations of observed calories from each of 4 predictions, including our own, as noted. The distributions of the individual deviations and the average fit of the 4 predictions are shown graphically in Figures 2, 3 and 4, and the average measurements and calculated rates in all groups of controls given in Table IV. It was not possible to separate the total comparison series into first and later tests since some of the reports gave data already averaged for the individuals. The Benedict prediction (in which metabolism is considered as a simple function of weight, disregarding height, and age in the range from 17 to 21) shows itself decidedly inferior to the other three by excessive scatter of individual rates about the mean. In both series of controls, according to this last prediction, rates run as low as  $-11$  or  $-12$ , and as high as  $+33$  and  $+35$ , whereas a much more reasonable total spread is indicated by each of the other three standards.

The general similarity of the distributions according to three of the four predictions suggests immediately the logic of making their zero-points coincide before any really fundamental comparisons are attempted. This we have done by applying the constant percentage corrections needed to center each of the older predictions for our standard data. On this more legitimate basis the rates have been recalculated for those individuals of both series of controls for whom data on first tests are separately available, since averaged measurements would not be strictly comparable with those of our standard series. This leaves a comparison series of 85 individuals. The standards have then been recompared as to: 1.—new algebraic average of individual rates; 2.—standard errors of estimating rates by the various predictions; 3.—percentiles falling within  $\pm 10$  per cent and within



**BASAL METABOLIC RATES**  
OF 97 NORMAL GIRLS 17 THRU 20 YEARS AT THE UNIVERSITY OF  
WISCONSIN CALCULATED BY DIFFERENT "NORMAL" PREDICTIONS  
*Around Baseline Anesthetics*

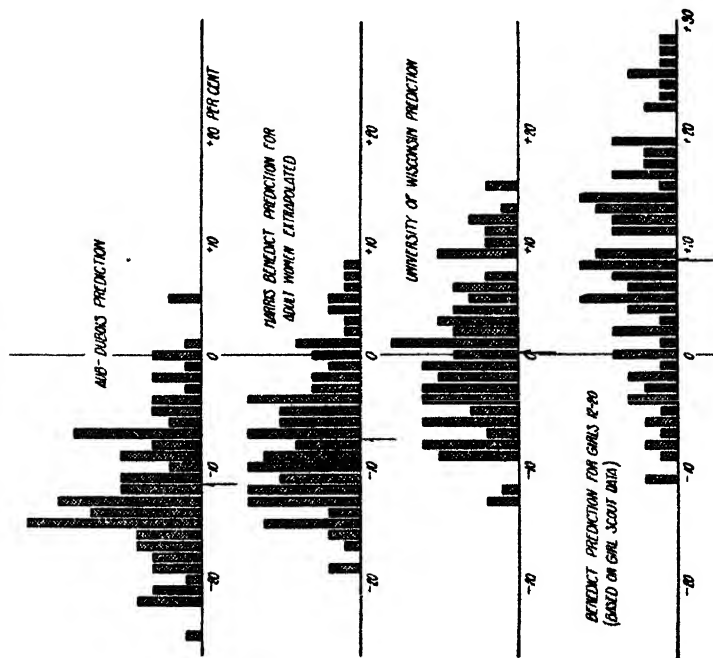


FIG. 2

**BASAL METABOLIC RATES**  
OF LATER TESTS ON 69 OF THE WISCONSIN STANDARD  
GROUP OF SUBJECTS  
*Around Inducement Anesthetics*

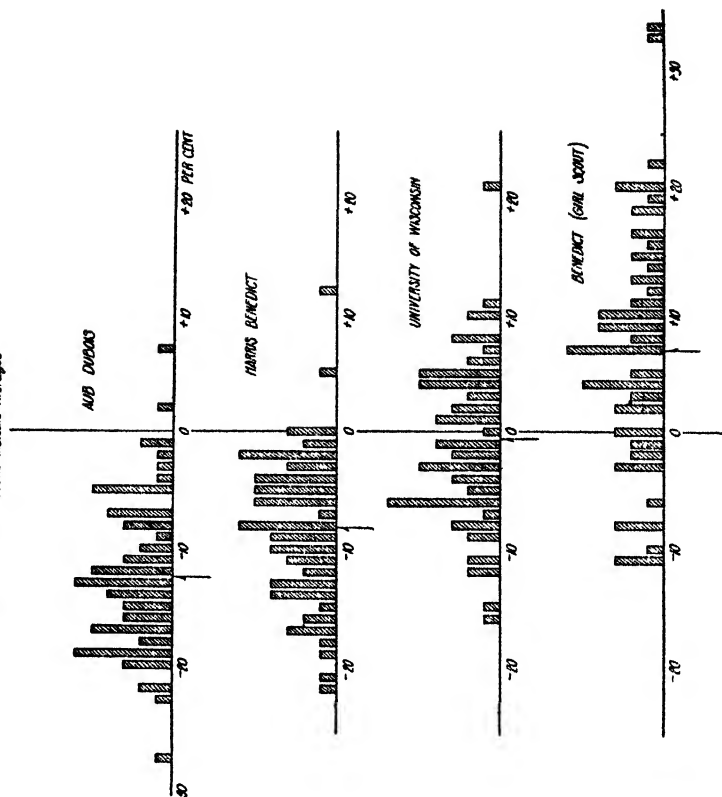


FIG. 3

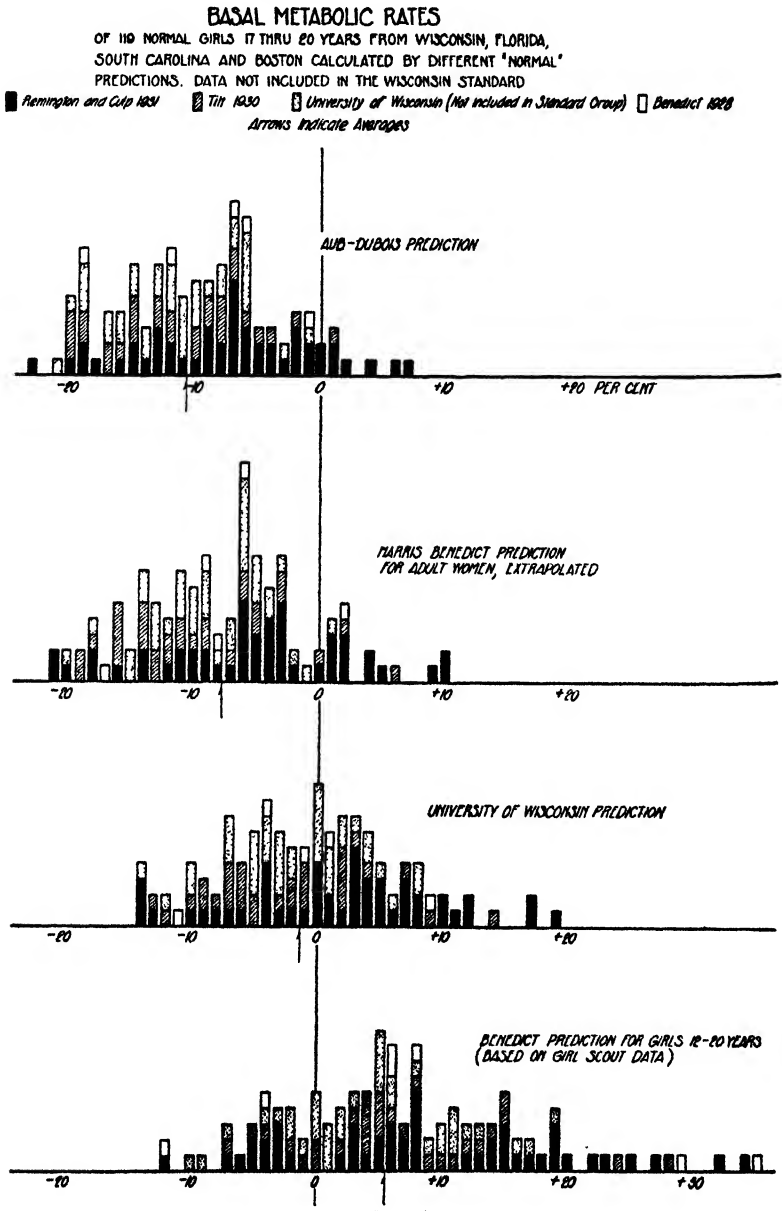


FIG. 4

$\pm 15$  per cent of the respective predictions; and 4.—extreme ranges of individual rates outside  $\pm 10$  per cent. The findings are summarized in Table V.

TABLE IV

AVERAGE MEASUREMENTS AND CALCULATED METABOLIC RATES IN DIFFERENT GROUPS OF NORMAL CONTROLS

(All tests each subject)

Source	Subjects	No. of subj.	No. of tests	Aver. Wt., kg.	Aver. Ht., cm.	Aver. cal./24 hrs.	Aver. cal./Sq.M./hr.	Aver. Metab. Rate acc. to Pred. of:			
								Aub-Du-Bois.	Harris-Ben., extrapolated.	Benedict, girls 12-20	Univ. Wis., girls 17-20
U. W. std. subj.	Univ. students	97	163	55.63	163.5	1296	34.01	-11.9	-7.6	+7.7	-0.3
U. W. suppl.: M.E.S.	Univ. students	7	7	56.9	165.8	1300	33.31	-15.0	-8.4	+5.0	-2.0
J. Fish Shipley	Do.	21	21	54.8	162.4	1266	33.61	-11.0	-9.0	+7.0	-1.8
	Do.	9	13	57.5	162.7	1297	33.54	-10.1	-7.8	+3.9	-0.2
Tilt, 1930	College students, Florida	29	*	53.4	159.4	1235	33.40	-11.6	-10.0	+6.5	-2.6
Benedict, 1928	Normals, Boston.	5	*	60.9	161.0	1336	34.04	-9.0	-7.6	+0.8	-1.2
Rem. & Culp, 1931	Stud. nurses, S. Carolina	48	138	56.1	162.9	1320	34.56	-8.1	-6.1	+8.5	-1.0

\* Each test reported = average of several observations.

By all the criteria there is very little choice between the Wisconsin prediction, the corrected Harris-Benedict and the corrected Aub-DuBois in success of predicting for both groups of controls, though in all-round accuracy of fit they arrange themselves consistently in the order named.

By each of the criteria the excessive spread of rates as figured by the Benedict prediction for girls is again brought out.

As a final test of the various standards, we were interested to find which would afford the best fit for the individuals of atypical body-build in the two series of controls. Superiority in this respect has been variously ascribed to the DuBois prediction (presumably because of the surface area concept included, and because it is not statistical in nature (19)), and to

TABLE V

COMPARISONS OF "FIT" OF DIFFERENT "NORMAL" PREDICTIONS OF METABOLIC RATES WHEN OLDER STANDARDS ARE CENTERED TO FIT AVERAGE U. W. DATA

(First tests only each individual)

Source	No.	Prediction	Algebraic aver. of rates	Std. Error of estim. rates	Of total rates within $\pm 10\%$ of pred.	Of total rates within $\pm 15\%$ of pred.	Extreme rates, (outside $\pm 10\%$ )
U. W. std. sub- jects—(tests used for std.)	97	Univ. Wis., Girls 17-20	(+0.1)	6.54	% 87	% 100	+15, -13
		H-B, Corr.*	(-0.2)	6.57	88	99	+16, -13
		DuBois, Corr.**	(+0.5)	7.13	84	98	+19, -15
		Ben., Corr.***	(+0.4)	8.67	74	89	+19, -18
Other controls: (U. W. Suppl: 37; Rem. & Culp: 48.)	85	Univ. Wis. Girls 17-20	+0.1	7.07	86	96	+19, -14
		H-B, Corr.*	+0.4	7.26	86	96	+18, -15
		DuBois, Corr.**	+3.1	7.96	84	95	+22, -13
		Ben., Corr.***	+0.02	8.90	75	91	+25, -19

\* Harris-Benedict extrapolated, corrected by  $-7\%$  to center for U. W. Std. series.

\*\* Aub-DuBois corrected by  $-12\%$  to center for U. W. Standard series.

\*\*\* Benedict's prediction for girls, 12-20, corrected by  $+8\%$  to center for U. W. Std. series.

either the Harris-Benedict or the Dreyer standards (because they *are* statistical in nature!) It is obvious that such comparisons can be legitimate only if the different standards start from approximately the same mean levels. For this comparison we picked out those individuals from our two test series who were judged to differ significantly in proportion of weight for height from the majority of their fellows. For convenience we did this by taking those points which fell outside (or just at the border of) the standard area delineated on Figure 1, and by fitting the data of the com-

parison series to the chart in the same way. By this method there were set apart 17 subjects in our group and 21 from the total comparison series. Of these, 25 were overweight for their height by from 15 to 47 pounds according to our standard regression equation, and by from 12 to 40 pounds by Bardeen's tables. The other 13 were underweight by 12 to 34 pounds by our formula, and by 13 to 35 pounds by Bardeen. The groups are not large enough to justify the calculation of statistical constants, but general comparisons involving average and extreme findings are interesting.

The average metabolic "rates" as calculated for both underweight and overweight groups were quite similar to the corresponding figures for the total groups when calculated according to either the original Aub-DuBois or Harris-Benedict predictions. Calories per square meter per hour averaged 34.49 for the two overweight groups, and 34.68 for the underweight, against a grand average for all controls of 34.11. Thus the burden of evidence suggests that these light- and heavy-weight sub-groups are not essentially abnormal in rates of heat-production when compared with the populations from which they were drawn.

The Benedict prediction for girls is at odds with these comparisons in showing distinctly different types of rates for the two types of body build. Thus for the over-weight girls the calculated rates turned out to be close to the "normal" expectation by this prediction, averaging  $-1.9$  in the Wisconsin group, and  $-2.1$  in the other group, with a total range of only  $+11$  to  $-14$ . On the other hand, the underweight girls consistently showed high rates, averaging  $+18$  in the Wisconsin group, and  $+26.8$  in the other group, with total range from  $+7$  to  $+35$ . The excellent average fit afforded by Benedict's standard for his own small group of predominantly overweight girls, alone among the groups included in the present study, is therefore not surprising (Table IV). Though this standard may be valid for girls between 12 and 17, which ages furnished the data it was based on, its extrapolation for subjects from 17 to 21 has by no means appeared justified. Its use in the clinic, either alone or in conjunction with other standards, has led to much confusion, which the above observations on normal girls help to explain.

When the older standards had been centered for the Wisconsin data, the results shown in Table VI were obtained.

Again there appears to be little choice between the Wisconsin, the corrected Harris-Benedict, or the corrected Aub-DuBois predictions, assuming that these subjects should show on the average essentially "normal" rates. There is a very slight advantage with the Wisconsin prediction in

smaller total range of individual rates if we consider all the groups compared. The Benedict prediction for girls has been improved by centering

TABLE VI  
COMPARISONS OF "FIT" OF DIFFERENT "NORMAL" PREDICTIONS OF METABOLIC RATES FOR OVERWEIGHT AND UNDERWEIGHT INDIVIDUALS FROM VARIOUS SERIES OF NORMAL CONTROLS WHEN OLDER STANDARDS ARE CENTERED TO FIT AVERAGE U. W. DATA  
(All tests each individual)

Source	No.	Prediction	Overweight		Underweight	
			Algebraic aver. of rates	Range of rates	Algebraic aver. of rates	Range of rates
U. W. std. subjects	Overweight: 10	Univ. Wis., Girls 17-20	+0.2	+12, -8	+1.5	+7, -4
		H-B., Corr.*	+1.6	+13, -8	+2.2	+10, -3
	Underweight: 7	DuBois, Corr.*	+0.9	+16, -11	-0.3	+6, -8
		Ben., Corr.*	-10.2	+1, -18	+7.9	+15, -1
Other control series**	Overweight: 15	Univ. Wis., Girls 17-20	-1.5	+9, -13	+4.2	+9, -6
		H-B., Corr.*	+0.9	+11, -13	+1.6	+9, -9
	Underweight: 6	DuBois, Corr.*	+2.9	+13, -9	+7.7	+14, -1
		Ben., Corr.*	-9.3	+0, -20	+17.4	+25, +7

\* Corrections given with Table V.

\*\* Overweight subjects: U. W. total supplementary series: 4; Tilt: 2; Remington & Culp: 6; Benedict: 3.

Underweight subjects: U. W. Supplementary series: 2; Remington & Culp: 4.

of course only to the extent of distributing the excessive scatter between over-weight and under-weight subjects. There would seem to be no need for considering this standard any longer in the presence of three others which appear superior to it by every criterion applied.

#### DISCUSSION

The success of predicting for these subjects by the methods of either of the classical adult standards would seem adequately to justify the classing of girls from 17 to 21 with adult women in the matter of the physiology of heat-production. This, Benedict was unwilling to do until more normal data should be accumulated. Though the original Nutrition Laboratory

series of 103 normals on whom the Harris-Benedict equation for women was based included data on 12 girls from 15 to 21, Benedict preferred to restrict the use of his tables to ages from 21 up, and thought it safer tentatively to bridge the then inadequately surveyed transitional years by extrapolating upward the curves obtained on his younger Girl Scouts (3, 4). The Harris-Benedict equation for adult women, extrapolated in a straight line through ages certainly down to 16, affords the most accurate of the three older standards for young women which we have tested.

Various modifications of the original Aub-DuBois or the Harris-Benedict predictions by correction of their constants as suggested from different sources (20, 7, 18) have not had to be considered as separate standards for the present study since the application of the more fundamental tests of fitness involves arbitrary readjusting of their mean absolute levels to make them strictly comparable.

The omission of calculations based on the Dreyer predictions (10) is admitted as regrettable since more and more laboratories appear to be using them with satisfaction. Being based upon the same original data, the mean predictions run essentially the same by the Dreyer and the Harris-Benedict standards. But the different types of statistical treatment given the data must affect the relative success of the two in predicting for individuals whose measurements vary much from the group average. According to Benedict, better success has been attributed by some to the Dreyer equations in predicting for individuals of unusual build, though his comparisons for such subjects among the 1928 series of normals from the Nutrition Laboratory (5) do not show this to be the case.

More recently Jenkins, of the University of Chicago, (17) has compared various predictions for adults and children of both sexes against different published bodies of control data and has concluded that the Dreyer formula using observed weight, as expressed in the tables of Stoner, possesses the most advantages and least disadvantages of any on the list. He bases this decision however on general considerations including simplicity and range of applicability, rather than consistent superiority in relative prediction accuracy by the statistical criteria which he himself sets up. By these purely objective criteria his tables show the Harris-Benedict to be decidedly superior for adult males, on whom the majority of available data has been compiled, while the comparative showings are inconsistent for women and the Harris-Benedict is not available for children. There are very few test data included for girls of the ages of the present study. The fact that women are more variable than men in bodily configuration, and

the definite correlation that exists between metabolism and the more typical measurement of height, suggest that further comparisons are necessary on data for women to justify a general decision in favor of the Dreyer prediction, which leaves the factor of height out of consideration.

The present study is in agreement with that of Jenkins in finding the Aub-DuBois prediction less accurate than the Harris-Benedict by the fundamentally important criterion of total spread of individual deviations expressed by the standard errors of estimate corrected to the same mean. Ultimate choice would logically depend upon whether one prefers the maximum possible accuracy in predicting for individuals, or the general biological significance inferred in some form of prediction using the surface area concept, even with the inevitable sacrifice of some degree of accuracy involved in estimating the surface itself. It seems doubtful that any one standard should be expected to meet universal needs.

#### SUMMARY AND CONCLUSIONS

The compiling of a statistically significant number of metabolism measurements on girls from 17 through 20 was undertaken at the University of Wisconsin for the purpose of deciding upon a valid standard of normality for such subjects. This small but important sex and age group represents a gap relatively unexplored by the older data upon which prediction standards still in general use were based. Diverse methods used for tentatively bridging this gap gave rise to predictions which are contradictory and confusing in practice.

Basal heat production was determined in 163 tests on 97 girls whose ages were approximately uniformly distributed over the 4 years from 17 through 20. These were University students who were classed as Grade A in their medical and physical examinations and who were judged by further special examination and questioning at the time of their tests to be free from indisposition or defects that should disqualify them from serving as physiological normals.

The results of the tests were subjected to various types of mathematical analysis and the first accepted tests on the 97 subjects were made the basis of a prediction standard expressing the equation: Basal heat production in calories per 24 hours =  $10.63 \times \text{weight in kg.} + 3.23 \times \text{stature in cm.} + 184.61$ . The equation was arrived at by standard methods of multiple correlation.

Comparisons by various criteria were made of the "fit" of this standard and that of the Aub-DuBois, the Harris-Benedict prediction for adult women, extrapolated for these ages, and of Benedict's special prediction



for girls from 12 through 20, when each was applied 1.—to the data of the Wisconsin series; and 2.—to a comparably large series of normal controls of similar ages collected from different observers and widely different localities. The latter included data on 77 young women studied in non-goiter-belt regions of the south. The fit of the different standards was similar for the two series of controls.

As they stand the Aub-DuBois prediction averaged from 8 to 15 per cent and the Harris-Benedict from 6 to 10 per cent too high, while the Benedict averaged from 1 to 8.5 per cent too low to describe the measured metabolism of the various sub-groups of controls tested.

Tests of fitness more fundamental than mere success in predicting average absolute level of heat-production for groups—i.e., standard errors of estimating the rates, percentiles falling within  $\pm 10$  per cent and  $\pm 15$  per cent of the respective predictions, and extreme ranges of calculated rates, were applied after the older standards had been put on a comparable basis with the Wisconsin standard by applying constant percentage corrections to center them for the Wisconsin data. Finally a special set of comparisons was made for the 38 individuals of the two test series who were atypical in proportion of weight to height.

The results of the various comparisons indicated little choice in accuracy of predicting for either group of controls by the Wisconsin prediction, the corrected Harris-Benedict, or the corrected Aub-DuBois, though by all the criteria taken together, they arranged themselves in the order named. The Benedict prediction compared with the other three showed excessive scatter of individual rates about their mean. According to this standard, but not judged by the others, the underweight girls of these series showed very different types of metabolic rates than the overweight girls.

The older standards should be modified only after many groups of data, covering all ages, can be compared to show the ultimate corrections needed to make them fit the largest possible number of cases. It is hoped that the present study can contribute toward an eventual agreement in this respect. Meanwhile, since the Wisconsin prediction has proved to be valid for these considerable numbers of normal girls examined with comparable technic in both the north and south of America, it is offered for its immediate interest in either clinical or physiological comparisons.

*Acknowledgments.*—The author wishes to acknowledge gratefully the coöperation of the students who served as subjects for the tests, and in addition to express her indebtedness for the following essential collaboration:

Normal girls of the desired ages were obtained as subjects through the staff of the Student Health Department of the University, and specifically the interest of Dr. William A. Mowry, Dr. Sarah I. Morris, and Dr. Irma Backe. The statistical portion of the work was made possible by the collaboration of Professor Mark Ingraham of the Department of Mathematics, and the extensive computations for the preliminary analyses and for the final prediction standard were performed by Miss Beatrice Berberich of the University Statistical Service. The work as a whole was suggested in the beginning by Dr. E. L. Sevringhaus of the Department of Medicine. Without his unfailing support and inspiration the project could neither have been undertaken nor completed.

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# THE PARALLEL DETERMINATION OF THE RESPIRATORY QUOTIENT AND ALVEOLAR AIR OF MAN IN THE POST-ABSORPTIVE CONDITION\*

By

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THE respiratory quotient as determined in short periods is often rendered of doubtful value because of the likelihood of abnormality in breathing. The uncertainty is greatest when, as is not uncommon, single, isolated periods are used as a base line for the study of the effect of a superimposed factor. In successive periods there is usually compensation for the possible over-ventilation in one period by an under-ventilation in a following period, so that the average of several periods made in succession is much to be preferred. When a substance is ingested, and there is a marked rise in the respiratory quotient, the measurements of the respiratory quotient and the gaseous exchange together are not sufficient to aid in interpretation of the results, even when carried out with a type of respiration apparatus in which there is every reason to believe that the breathing is perfectly normal. There exists the possibility of the formation of organic acids in the intermediary metabolism of the material which has been ingested, which may alter the carbon dioxide content of the blood. Ideally, one should have frequent determinations of the carbon-dioxide tension of the arterial blood in order to determine whether the changes in the respiratory quotient are the results of metabolic activities alone or are the results of concomitant changes in the carbon-dioxide content of the blood. However, this is very rarely attempted and cannot be easily performed in humans without disturbing seriously the measurements of the respiratory exchange and their interpretation. It has frequently been shown that the carbon-dioxide tension of the arterial blood and the carbon-dioxide tension of the alveolar air run parallel in normal subjects and are nearly identical, so that the simultaneous determination of the alveolar carbon dioxide ought to throw light upon the normality of the breathing and upon the significance of the respiratory quotients as found.

\* A preliminary communication was presented at the annual meeting of the American Physiological Society, Montreal, April, 1931, *Amer. Jour. Physiol.*, 1931, 97, 509.

The use of the alveolar air as a control upon the normality of the respiratory exchange has been employed by Löb (9), Hindmarsh (5), Marsh (11), Schill and Weiss (14) and Heckscher, Faddersböll and Mogensen (3). Ideally, one should make the measurements of the respiratory exchange and of the composition of the alveolar air simultaneously. This has been done by several workers. Lindhard (8) collected by hand the alveolar air at the end of each expiration. Krogh and Lindhard (7) had an electric automatic arrangement for the collection of alveolar air. Löb (9), Henderson and Haggard (4), Wright and Kremer (15), Regelsberger (13), Marschak (10), and Olivier and Bretey (12) have used various automatic methods or methods employing intermittent collection by hand for obtaining samples of the alveolar air during the periods of measurement of the respiratory exchange. Many of these have given results which were generally lower than the samples obtained by the Haldane-Priestley method. After we had begun our studies, Heckscher, Faddersböll, and Mogensen (3) published their method of obtaining a sample of the alveolar air from a Krogh (6) closed-circuit respiration apparatus with a simultaneous graphic record of the respiration. We have measured the respiratory exchange by an open-circuit arrangement with mouthpiece and at the same time samples of the alveolar air have been collected by the Haldane-Priestley method by a special arrangement. The problem at hand was to determine the course of the alveolar air in connection with the ingestion of small quantities of sugars, with respect to their effect upon the gaseous exchange, particularly the respiratory quotient as compared with findings in experiments in which no dose was given. This paper deals with the results obtained in experiments in the post-absorptive condition that were used as controls for the series in a following paper. As the experiments with the sugars extended over several hours, it was necessary to know what would be the course of the respiratory quotient and of the alveolar air during the same periods of time when no sugars were ingested.

*The Respiratory Exchange and Alveolar Air in the Post-absorptive Condition*

*Method of determining the respiratory exchange.*—The respiratory exchange was measured by an apparatus which was used in another study and reported elsewhere (Carpenter and Fox, 1).

*Method of collecting alveolar air samples.* The special feature of this study was the parallel collection of samples of alveolar air without interrupting the measurement of the respiratory exchange. The alveolar air was sampled according to the Haldane-Priestley principle at the end of a

normal expiration by means of the arrangement shown in Figure 1. A quick-seating valve, *A*, was inserted at a point on the inspiratory side closely adjacent to the mouthpiece, *D*, between the inspiratory and expiratory valves, *B* and *C*, of the system for determining the respiratory exchange. On the expiratory side of the mouthpiece, *D*, was soldered a small copper tube, bent downwards. At the lower end of the copper tube

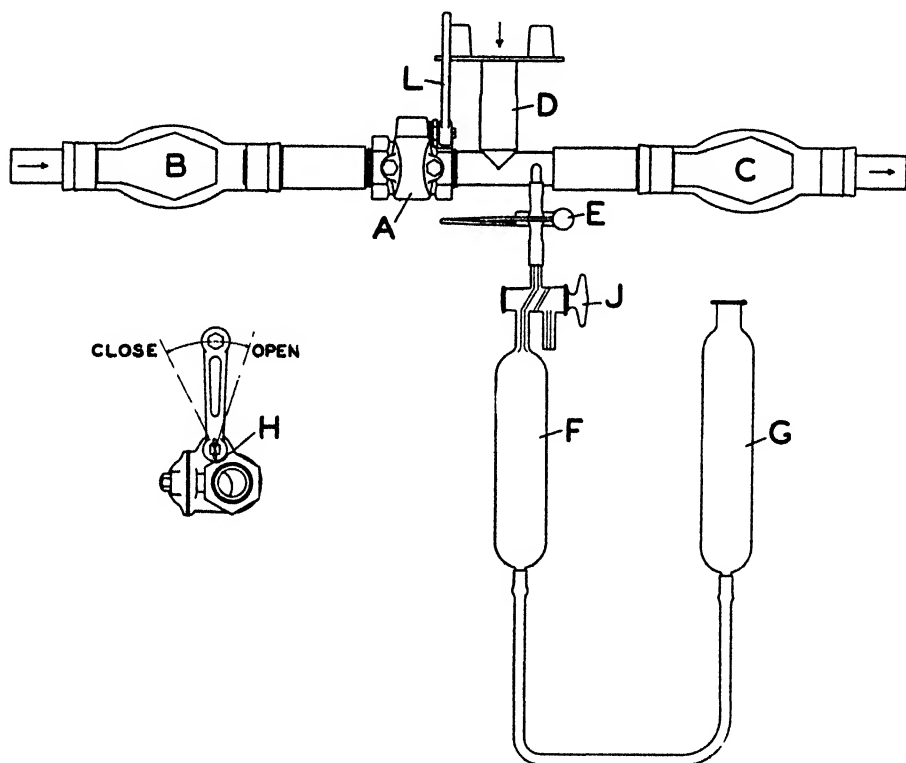


FIG. 1. Arrangement for sampling of alveolar air during the measurement of the respiratory exchange of man. *A*, quick-seating valve between inspiratory and expiratory valves, *B* and *C*. *D*, mouthpiece; *E*, spring pinchcock; *F*, gas sampler with mercury levelling bulb, *G*; *H*, cross section of quick-seating valve; *J*, stopcock; *L*, handle of quick-seating valve, *A*.

was attached a rubber tube with a spring pinchcock, *E*, and a gas sampler, *F*, with its mercury levelling bulb, *G*. A cross-section view of the quick-seating valve, *A*, is shown at *H* with the handle midway between open and closed. The procedure of taking a sample of the alveolar air was as follows: The operator first filled the gas sampler with mercury to above the capillary above the stopcock, *J*, and attached the sampler to the rubber tube with pinchcock, *E*. The stopcock, *J*, was then closed and the spring pinch-

cock, *E*, opened. The operator then watched the breathing as it was recorded on the kymograph. After several respirations had been recorded and it was certain that there was no alteration in the rate or character of the respiration, the operator then gave the signal "blow" to the subject at the end of a normal expiration, at the same time closing the quick-seating valve, *A*, by means of the handle, *L*. When it was seen that the subject had come to the end of a forced expiration, the operator opened the spring pinchcock, *E*, and lowered the mercury levelling bulb, *G*. As soon as the sampler was filled with the alveolar air the operator closed the cock, *J*, signalled the subject "all right," opened the quick-seating valve, *A*, and the subject took a deep inspiration and then breathed as required to recover and continued as normal. During the taking of the sample, 2 to 3 seconds, the subject had held the volume of air in his lungs constant according to instructions which had been given him. The spring pinchcock,

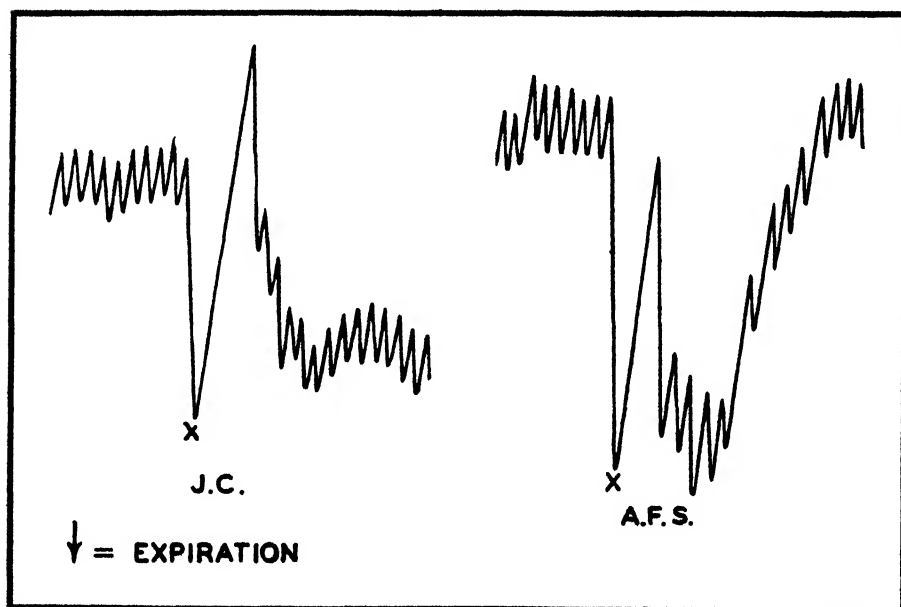


FIG. 2. Graphic record of respiration of subjects J. C. and A. F. S. during the taking of a sample of the alveolar air. Downward stroke is expiration and samples of the alveolar air were drawn at the point, *X*, while the subject kept the volume of the respiratory tract constant.

*E*, was then closed, the gas sampler detached and another attached in place. The alveolar air samples were analyzed by the Haldane portable gas analysis apparatus. The quick-seating valve prevented inspiration, if attempted, during the taking of the sample, and the whole procedure took

only a few seconds, during which the volume of air in the lungs remained constant. The whole volume of the forced expiration with exception of the amount taken by the sampler was thus a part of the ventilation of the respiratory tract, and was compensated for by the shallowness of the breathing following the collection; thus there was no disturbance in the measurement of the total respiratory exchange. Preliminary trials with this arrangement with two subjects showed that samples could be taken at least every 15 minutes without disturbing the uniformity of the results of the measurements of the total respiratory exchange, and later it was found that samples could be taken every seventh or eighth minute without affecting the total respiratory exchange. A graphic record of the taking of samples of alveolar air with the two subjects used in the experiments is shown in Figure 2. The advantage of this method is that the sample can be obtained as part of the regular respiration experiment. Also, that by our method of measuring the respiratory exchange a graphic record is obtained of the breathing before and at the time the alveolar sample is drawn. It is thus possible to detect changes in the type of breathing due to anticipating the sample or the starting of inhalation before drawing the sample.

#### *The Respiratory Quotient of Expired Air*

J. C., a subject whom we have used for many years, was entirely accustomed to the apparatus. The respiratory quotients of this subject for individual periods for each experiment are plotted in Figure 3. As the entire series of experiments was post-absorptive, one would expect that the course of the respiratory quotients during the morning hours would be practically without change and that there would be little variation from period to period, provided the subject sat quietly and was uniformly awake. However, the respiratory quotients with J. C. in the first four fifteen-minute periods were usually at a slightly different level than for the groups of periods succeeding the intermission. In general, with J. C. the 9 to 10 periods after the preliminary hour of 4 periods were slightly lower or slightly higher than the average of the preliminary hour and more uniform than during the first hour. All the experiments were begun immediately after insertion of the mouthpiece but after a half hour's rest, sitting quietly. Even with this subject, who had had long experience with breathing appliance apparatus, a period of adjustment was required before the respiratory exchange became stable. The cause for the shifting of the level of the respiratory quotient after the intermission is not known.



The other subject, A. F. S., had had little previous training. He was familiar with the procedure and had seen many experiments of this type, as he had previously been an assistant in the laboratory.

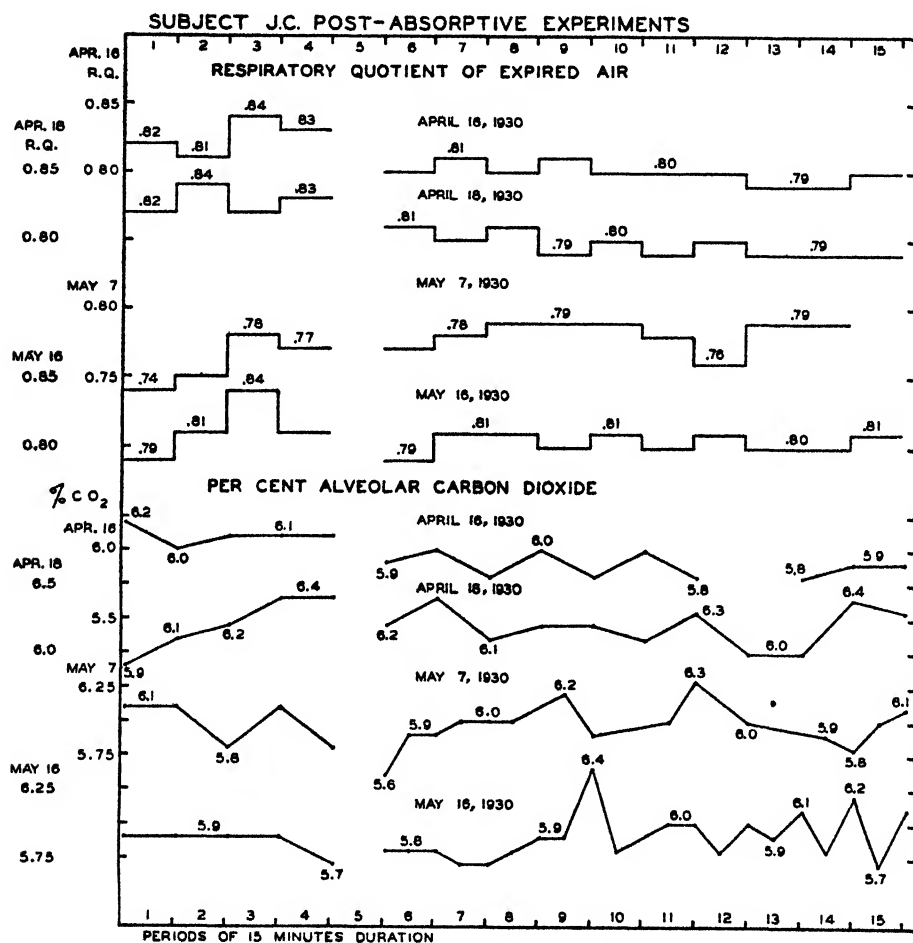


FIG. 3. The respiratory quotient of expired air and the percentage of alveolar carbon dioxide for the individual experiments with J. C. in the post-absorptive state. Period 5 is the interval during which the mouthpiece was taken out corresponding to the later experiments with sugar when the dose was given.

The respiratory quotients for the individual experiments with A. F. S. are plotted in Figure 4. On all three days the initial quotient was low with a gradual rise during the three succeeding periods. The quotients of the first period show strikingly the necessity for a period of preliminary

breathing before beginning the measurements. The period required was longer with A. F. S. than with J. C. The course of the respiratory quotient with A. F. S. was extremely variable, not only during the preliminary hour, but also in the periods following an intermission. As these periods

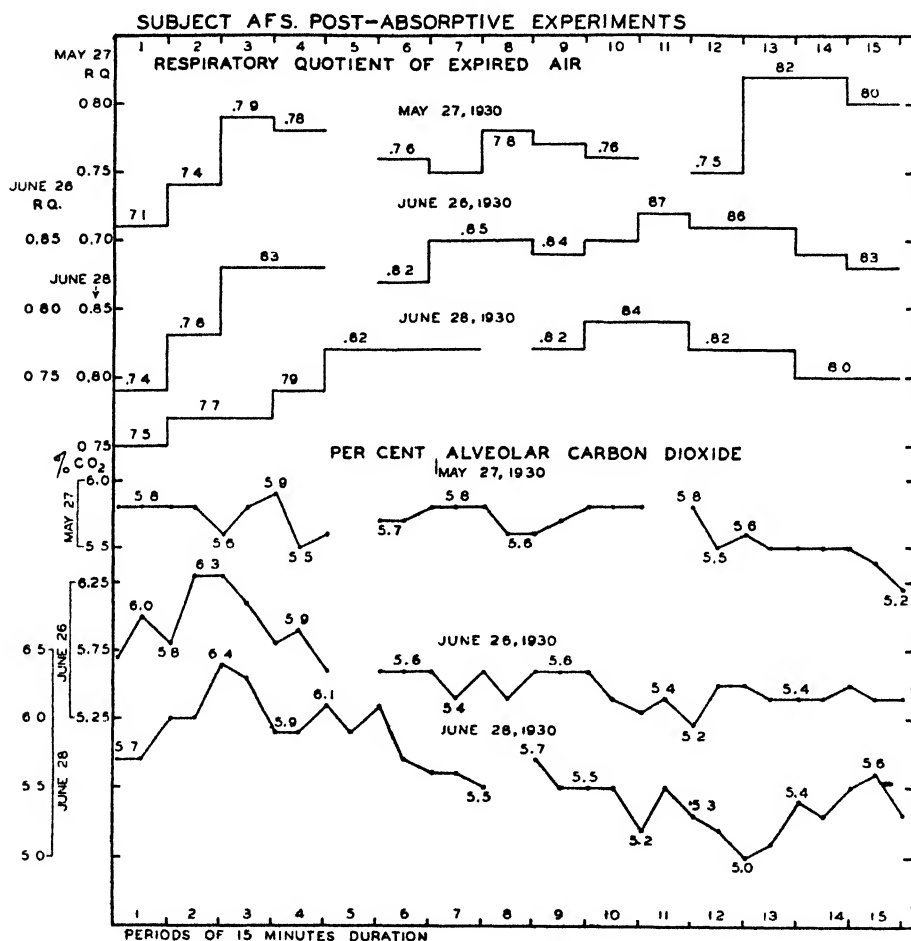


FIG. 4. The respiratory quotient of expired air and the percentage of alveolar carbon dioxide for the individual experiments with A. F. S. in the post-absorptive state.

were all post-absorptive, it is difficult to explain the wide variations, but other subjects (details unpublished) have shown similar characteristics of respiratory exchange. We may regard the first hour as one of adjustment, and the succeeding periods as evidence of instability of the regulatory mechanism with a breathing appliance, or evidences of fatigue.

*Percentage of Alveolar Carbon Dioxide*

The percentage of alveolar carbon dioxide was determined in parallel with the respiratory exchange at the beginning of the series in this investigation only once during a 15-minute period. Subsequently it was determined every 7 or 8 minutes throughout the experiments. The determinations for the experiments for J. C. are plotted in Figure 3. There is no apparent relation between the variations in the respiratory quotient of the expired air and the percentage alveolar carbon dioxide. In general with J. C. there was no marked tendency for the alveolar air to change in level throughout a total of 14 periods of one-quarter hour each during the morning. There were irregularities from time to time but these are not extreme and probably not greater than one would expect when each determination represents the collection of a single sample and all values are included.

The series with A. F. S. are plotted in Figure 4. On May 27, for the most part, the range was not wide and there was very little change in level until the twelfth to thirteenth periods when there was a drop from 5.8 to 5.5 per cent. The experiment ends with a value of 5.2. On June 26 the preliminary values were higher than on May 27, with a general level of 5.9. After the period of intermission the values were all on a much lower level than in the preliminary hour. This corresponds to the generally higher average respiratory quotient which was found during this period of time. On June 28 the majority of the values were at a normal level, 5.7 to 6.4, during the first consecutive series. However, beginning with period 5, there was a drop with a value of 5.5 at the end of period 7. In general, the rest of the alveolar carbon dioxides were lower than in any of the preceding periods, a minimum of 5.0 per cent being reached. There is a relationship between the low percentages of alveolar carbon dioxide and the generally higher respiratory quotients during this period of time. This subject therefore was irregular both with respect to the respiratory quotient of the expired air and with respect to the alveolar carbon dioxide. In general, there was a tendency for his alveolar carbon dioxide to become lower as the experiments proceeded. This is shown more definitely in the average of all respiratory quotients and the average of the alveolar carbon dioxide which are considered later.

*Average Respiratory Quotient of Expired Air*

The average respiratory quotient of the expired air for all of the experiments for J. C. and A. F. S. are plotted in Figures 5 and 6. With J. C. there

was practically no change in the average respiratory quotient for 14 periods of 15 minutes each during the morning. For A. F. S. the average of the initial period of the first hour was 0.73, an extremely low value for a subject even in a post-absorptive condition. This represents his adjustment to the apparatus and to the condition of the experiment, although

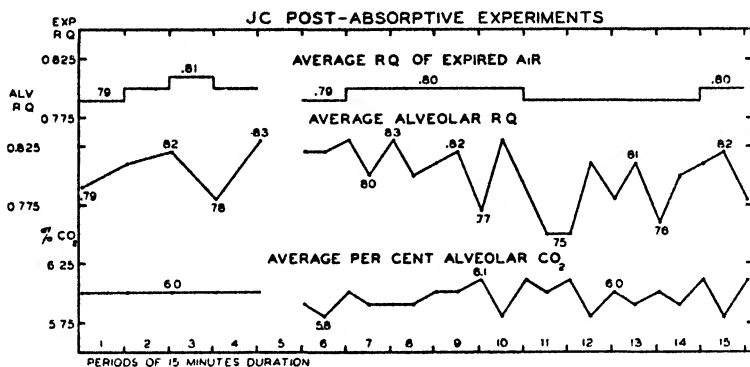


FIG. 5. Average respiratory quotient of expired air, average alveolar respiratory quotient, and average percentage of alveolar carbon dioxide in post-absorptive experiments with J. C.

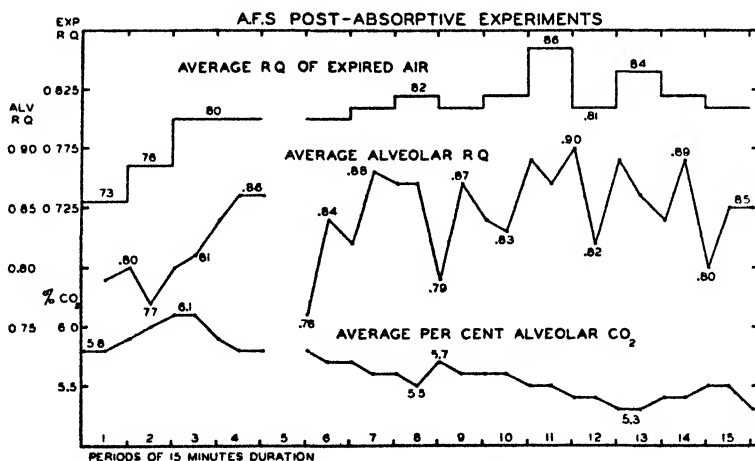


FIG. 6. Average respiratory quotient of expired air, average alveolar respiratory quotient, and average percentage of alveolar carbon dioxide in post-absorptive experiments with A. F. S.

the subject had rested the usual half hour, and measurements were begun at the end of the rest period as soon as the mouthpiece was inserted. The quotient of the next two periods was 0.76 and 0.80. After an intermission of 15 minutes, the average respiratory quotient for A. F. S. showed but little change for 5 successive periods. There was then an abrupt rise to

0.86 with a fall again to 0.81 with varying results to the end of the experiment, when it is again 0.81. The value of 0.86 is the average of 2 experiments instead of 3 for all the other periods. If a value of 0.75 is interpolated for the experiment of May 27, the average of this period (11) becomes 0.82, which is nearly the same as the values in periods 10 and 12. The course of the respiratory quotient with A. F. S. for fourteen periods during the morning showed a slight gradual rise until the maximum was reached, with a subsequent fall. The rise in respiratory quotient was accompanied by a fall in alveolar carbon dioxide so that the quotients were not true metabolic quotients.

#### *The Average Alveolar Respiratory Quotient*

The average alveolar respiratory quotient for J. C. is plotted in Figure 5 and for A. F. S. in Figure 6. The range for J. C. in the preliminary hour was from 0.78 to 0.83, and for the 10 periods following an intermission the range was from 0.75 to 0.83. With A. F. S. the fluctuations were somewhat larger as the quotients ranged from 0.76 to 0.90 with a tendency to follow the same course as the quotients of the expired air but at a generally higher level.

#### *Average Percentage Alveolar Carbon Dioxide*

The average alveolar carbon dioxide by periods for the experiments with J. C. is plotted in Figure 5 and for A. F. S. in Figure 6. During the preliminary or base-line hour there was no change in the average alveolar air with J. C. Subsequently, there was a slight drop and, for the remainder of the experiment, the values ranged between 5.8 and 6.1 per cent. Thus there was practically no change in the course of the alveolar carbon dioxide with J. C. for a period of three and one-half hours during the morning.

With A. F. S. the first hour showed a rise from 5.8 to 6.1 during the first three quarter-hours with a subsequent fall to 5.8. After the intermission the changes in general correspond nearly inversely to the changes in the respiratory quotient. The maximum respiratory quotient came in period 11 when the alveolar carbon dioxide was 5.5. Again a rise in quotient occurred in period 13 with a concurrent fall in alveolar carbon dioxide. With A. F. S. both the respiratory quotient and the alveolar carbon dioxide showed changes in the course of the morning which were not consistent with what one would expect in a series of periods in the post-absorptive condition. There is no theoretical reason for a rise in the respiratory quotient in the course of three hours and a half in the morning or for a fall in

the alveolar carbon dioxide when a subject is in a post-absorptive condition after an ordinary mixed diet.

*Relation of Alveolar Respiratory Quotients to Alveolar Carbon Dioxide*

If the sampling of the alveolar air is made incorrectly, the resultant carbon dioxide content of the alveolar air may be too low or too high according to whether there was a dilution of the alveolar air by too deep an inspiration or a retention of carbon dioxide due to too shallow breathing at the instant of taking samples. If there is over-ventilation or the respiratory center responds to a lower level of carbon dioxide, the carbon dioxide will be lower than normal and probably the respiratory quotient of the alveolar air will be too high because it is easier to "wash out" carbon dioxide by over-ventilation than to increase the oxygen absorption. Although the range of the per cent of carbon dioxide of alveolar air with J. C. was narrow (5.7-6.1), there were some values outside these limits. What is the relation between the fluctuations in the percentage alveolar carbon dioxide and the alveolar respiratory quotient? If the above reasoning is correct, when the carbon dioxide content of alveolar air is low, the alveolar respiratory quotient should be high, and vice versa. This assumes that the sampling is correctly made, that the expiration had been deep enough to obtain the real alveolar air, and that the analyses were correct. At the beginning of this work it was the intention to obtain the carbon dioxide only, and owing to the pressure of other work at the same time, duplicate analyses were not made of the samples obtained from the alveolar air. Whenever there was a value which was markedly aberrant the analysis was repeated. The object of the study was to obtain the general trend of the alveolar air, particularly the carbon dioxide in relation to the simultaneous changes in respiratory quotient of the expired air.

In Figure 7 are plotted the relationships between the alveolar carbon dioxide and the alveolar respiratory quotient for the two subjects. The range in alveolar respiratory quotients with J. C. was not wide, yet in spite of this there is a definite relationship between the respiratory quotient and the alveolar carbon dioxide content. As the carbon dioxide content falls, the respiratory quotient rises. This finding probably represents the slight variations in depth of respiration immediately preceding the taking of the sample. It must be recalled that only one sample was taken and this was at the end of a normal expiration. It is surprising that even with a narrow range of carbon dioxide content the relationship is evident.

With A. F. S., the values were scattered, but there was a tendency to a

relationship between the alveolar respiratory quotients and the alveolar carbon dioxide content. The relationship is not so marked as with J. C. This is in line with the variability of the respiratory center of this subject. There is a lack of uniform response and consequently a relationship between various factors in breathing will not be so constant with this subject

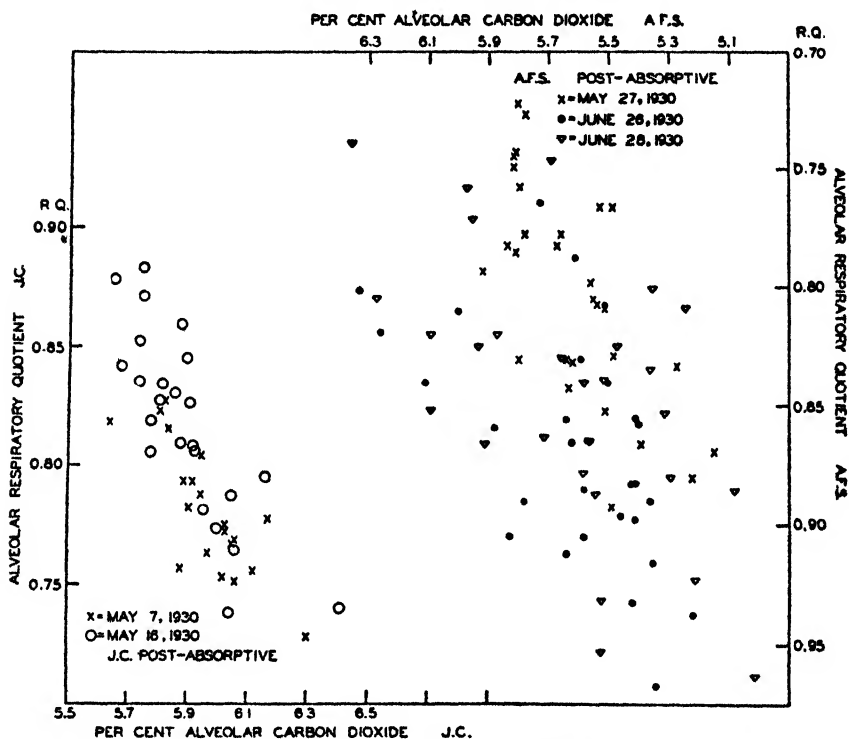


FIG. 7. Diagram showing relationship between percentage alveolar carbon dioxide and alveolar respiratory quotient for J. C. and A. F. S. in post-absorptive experiments. The experiments with A. F. S. are plotted in an inverse manner to those of J. C.

as with J. C., with whom all of the factors of ventilation and respiratory exchange were comparatively more consistent (unpublished material).

#### *Relation between the Alveolar Respiratory Quotient and the Alveolar Oxygen Deficit*

The respiratory quotient of the alveolar air is calculated in the same manner as that of the expired air, that is, the increase of carbon dioxide is divided by the oxygen deficit of the alveolar air. The relation between the alveolar respiratory quotient and the alveolar carbon dioxide is more apparent in the extremes than in the majority of the measurements. There-

fore one would expect from the mathematical derivation of the respiratory quotient that the oxygen deficit would bear an inverse relationship to the respiratory quotient just as it does in the main in the expired air when a subject is at rest (Carpenter and Fox, 2). It can be argued that with a nearly constant alveolar carbon dioxide percentage the alveolar respiratory quotient must vary with the alveolar oxygen deficit even if the analyses

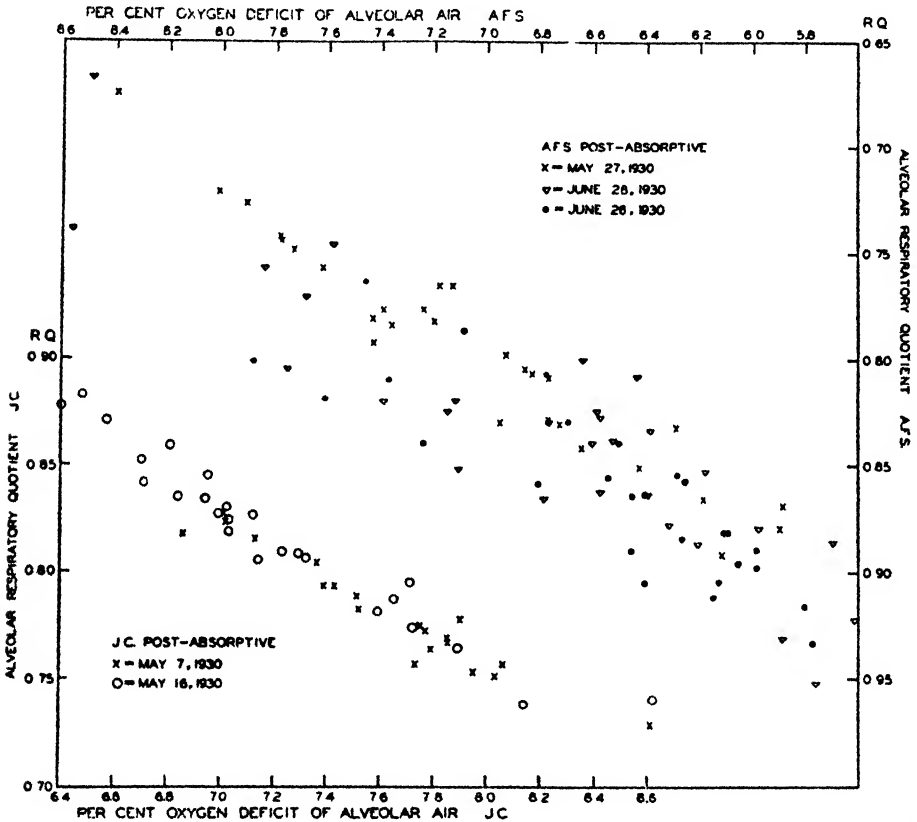


FIG. 8. Diagram showing relationship between the percentage alveolar oxygen deficit and the alveolar respiratory quotient for J. C. and A. F. S. in post-absorptive experiments. The experiments with A. F. S. are plotted in an inverse manner to those of J. C.

were wrong. This cannot be denied in our results because only one analysis was made of each sample, but the apparatus were standardized each day with outdoor air, and repetition of doubtful results usually gave agreeing results.

The percentages of alveolar oxygen deficit in relation to the alveolar respiratory quotients for J. C. and A. F. S. have been plotted in Figure 8.



In spite of the narrow range of the majority of the respiratory quotients, there is a very marked relationship between the alveolar oxygen deficit and the alveolar respiratory quotient in both experiments with J. C. Therefore with J. C. the alveolar respiratory quotients varied inversely as the alveolar oxygen deficits in the post-absorptive state.

With A. F. S. there was a noticeable variation in the alveolar oxygen deficit in relation to the alveolar respiratory quotient, although there is unquestionably a relationship as a whole. In all three experiments there were values of either alveolar oxygen deficit or alveolar respiratory quotient so aberrant that they are outside the limits of Figure 8. Even though there was no metabolic process which would result in a marked change in the respiratory quotient of the expired air, there was a definite relationship between the alveolar oxygen deficit and the alveolar respiratory quotient, that is, the lower the alveolar oxygen deficit, the higher was the respiratory quotient of the alveolar air.

*Correlation Coefficients between the Alveolar Respiratory Quotient and the Alveolar Carbon Dioxide and Oxygen Deficit*

The correlation coefficients between the alveolar respiratory quotient and the alveolar carbon dioxide and between the alveolar respiratory quotient and the alveolar oxygen deficit are given in Table I. The average alveolar respiratory quotients of J. C. were close to the average respiratory quotients of the same dates. The average alveolar respiratory quotients of A. F. S. are 0.039 and 0.033 higher on June 26 and 28 than the average respiratory quotients of the expired air on the same dates. The alveolar quotients of A. F. S. are in general more variable and higher than those of J. C.

The correlation coefficients between the alveolar carbon dioxide and the alveolar respiratory quotient were negative in all the experiments and more than 6 times the probable error in all but one (June 28, A. F. S.). There is therefore a definite relationship between the alveolar carbon dioxide and the alveolar respiratory quotient, that is, the higher the alveolar carbon dioxide, the lower the respiratory quotient. The relationship shown in Figure 7 therefore becomes much more evident when expressed mathematically. It is surprising that the relationship is so marked with J. C. when the relatively narrow ranges of both the alveolar carbon dioxide and respiratory quotient are considered. The lower correlation coefficients between these factors for A. F. S. are in part due to the irregularities in breathing and irregularities in the alveolar respiratory quotients.

TABLE I  
CORRELATION COEFFICIENTS BETWEEN THE ALVEOLAR RESPIRATORY QUOTIENTS AND THE ALVEOLAR CARBON DIOXIDE AND OXYGEN  
DEFICIT IN NO-DOSE EXPERIMENTS

Subject and date	Number of determinations	Alveolar R.Q.		Alveolar CO <sub>2</sub>		Alveolar O <sub>2</sub> deficit		Correlation coefficients	
		Average	S.D.	Average	S.D.	Average	S.D.	Alveolar R.Q. and alveolar CO <sub>2</sub>	Alveolar R.Q. and alveolar O <sub>2</sub> deficit
								r	P.E. <sub>r</sub>
1930				%		%		r	P.E. <sub>r</sub>
J.C.									
May 7	20	0.780 ± 0.022	0.026	5.97 ± 0.11	0.14	7.63 ± 0.34	0.42	-0.801 ± 0.054	-0.964 ± 0.011
May 16	25	0.817 ± 0.031	0.038	5.89 ± 0.12	0.16	7.20 ± 0.42	0.52	-0.808 ± 0.047	-0.967 ± 0.009
A.F.S.									
May 27	31	0.786 ± 0.049	0.064	5.64 ± 0.16	0.19	7.20 ± 0.62	0.80	-0.631 ± 0.073	-0.979 ± 0.005
June 26	30	0.873 ± 0.043	0.056	5.61 ± 0.21	0.27	6.43 ± 0.49	0.63	-0.537 ± 0.088	-0.894 ± 0.025
June 28	30	0.839 ± 0.047	0.063	5.63 ± 0.27	0.33	6.73 ± 0.63	0.80	-0.518 ± 0.090	-0.906 ± 0.022

The correlation coefficients between the alveolar respiratory quotients and the alveolar oxygen deficits were all negative and very marked. It should be remarked that analytical errors in the determination of the oxygen content of the alveolar air would bring about a good correlation between the respiratory quotient and the oxygen deficit when the range of the alveolar carbon dioxide is narrow, for mathematically it must follow that as the oxygen deficit falls off the respiratory quotient rises. A good correlation between the oxygen deficit and the respiratory quotient should never be taken as an index of the reliability of the analyses, as this must rest on other grounds.

These highly negative coefficients are indication of variations in the alveolar quotients due to metabolic variations in the respiratory exchanges in the same manner as the reciprocal relationships of the respiratory quotient and oxygen deficit of the expired air. It is recognized that not all of the variations with A. F. S. were truly metabolic variations, but part of them were due to shifts in the respiratory quotients accompanied by shifts in the general level of the alveolar carbon dioxide.

#### SUMMARY

The respiratory exchange of two human subjects was measured on 3 and 4 days for 4 consecutive 15-minute periods, and then for 9 or 10 consecutive 15-minute periods in the post-absorptive condition, in a sitting position, by means of an open-circuit respiration apparatus with mouthpiece as a breathing appliance. The alveolar air was sampled at least every 15 minutes without interrupting the measurements of the respiratory exchange, by means of an arrangement described in detail.

With one trained subject the average respiratory quotient and the alveolar carbon dioxide did not change significantly in the course of a total of three and one-half hours. With one untrained subject both the respiratory quotient and the alveolar carbon dioxide varied, usually in opposite directions during the same length of time. The alveolar respiratory quotients of both subjects tended to run parallel with the respiratory quotients of the total expired air.

There was a tendency to an inverse relationship between the alveolar carbon dioxide and the alveolar respiratory quotients which was more marked in the extreme values, and with the trained subject than with the untrained subject and was due either to slight variations in ventilation at the time of taking of the alveolar air samples or to variations in the sensitivity of the respiratory center.

There was a marked negative correlation between the alveolar respiratory quotient and the percentage alveolar oxygen deficit.

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# THE EFFECT OF GLUCOSE AND OF FRUCTOSE ON THE HUMAN RESPIRATORY QUOTIENT AND ALVEOLAR AIR\*

By

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THE effect of the ingestion of glucose on the respiratory exchange of animals and man has been determined repeatedly and a well established fact in the physiology of metabolism is that the respiratory quotient usually rises when the sugar is ingested. It is logical to assume that the increase in respiratory quotient is the result of the increase in the carbohydrate combustion because glucose is a constituent of many nutrients. However, that organic acids may be formed in the intermediary metabolism is by no means excluded because of the repeated results which have been obtained, or because it seems logical that there should be an increase in the carbohydrate combustion after the ingestion of glucose.

Practically all of the investigations with human subjects on the effect of the ingestion of fructose on the respiratory quotient in short periods show that there is a marked and prompt rise in the respiratory quotient. As long as this respiratory quotient was not over unity, it has been considered by many that there was an increase in the catabolism of carbohydrate as a result of the ingestion of fructose. In a study reported previously from this laboratory (Carpenter and Fox, 3), the effects of small quantities, 5 to 50 grams, were studied with respect to the respiratory quotient and the total metabolism. It was found that a marked rise occurred whether a small or large quantity was used and the main variation was the height to which the respiratory quotient rose and the length of time it remained above the pre-ingestion level. As the respiratory quotient will rise to over unity when larger quantities are given, it was postulated that the reaction was one of transformation of carbohydrate to fat, and that this took place regardless of the quantity ingested. No studies have been carried out in which the respiratory quotient and simultaneous de-

\* A preliminary communication was presented at the annual meeting of the American Physiological Society, Montreal, April, 1931. *Amer. Jour. Physiol.*, 1931, 97, 509.

terminations of the alveolar air directly have been obtained. It is of importance to know whether the changes in respiratory quotient due to glucose and to fructose are the results of metabolic processes only, or whether they are the results of combustion plus the influence of acids which may be formed in the intermediary metabolism of the sugars and lead to the expulsion of carbon dioxide from the blood with a fall of the carbon dioxide content of the blood.

The possibility of the formation of organic acids and of changes in the reaction of the blood in the intermediary metabolism of glucose and of fructose and their relation to the respiratory exchange have been studied in several ways. Taistra (12) determined the  $\text{CO}_2$ -combining power of the blood plasma of a dog in the 4 hours after the ingestion of 50 grams of glucose and found no significant change as compared with the pre-ingestion values. Moraczewski and Lindner (9) found a greater excretion of lactic acid in the urine of patients after the intravenous injection of 50 grams of fructose in 50 cc. of distilled water than after injection of glucose. Weiss and Klein (13) determined with narcotized rabbits the respiratory exchange and carbon-dioxide content of the blood at intervals after the ingestion by stomach tube of 15 to 30 grams of glucose and found no significant alteration in the carbon dioxide of the blood. Douglas and Priestley (5) found that the alveolar carbon dioxide remained practically unchanged after the ingestion of 75 and 80 grams of cane sugar. Gigon (6) measured the pH of the blood of men after the ingestion of 100 grams of glucose and found a maximum fall from 7.3 to 7.0 in 45 minutes. He also obtained similar results with rabbits. He found a lowering of the pH of the blood in man equivalent to 0.3 after the ingestion of 100 grams of fructose in 300 cc. of water. Lanyi (8) found that the administration of dextrose to dogs or man did not result in an increase of lactic acid in the blood, but the ingestion of fructose was followed by an increase in the lactic acid content of the body lasting 3 hours and returning to normal after 6 hours. Katayama (7) found an increase in lactic acid of the blood simultaneous with the hyperglycemia which took place after the ingestion of 1.75 grams of dextrose per kilogram of body weight in 22 normal and diseased subjects. Oppenheimer (10) found no increase in lactic acid in the blood of normal persons or of patients with diseases of the liver when 50 grams of glucose or of fructose in 300 cc. of water were taken by mouth. Campbell and Maltby (1) found no alteration in the  $\text{CO}_2$ -combining power and lactic acid content of the blood in man as the result of the ingestion of either 100 grams of glucose or of maltose, but the ingestion of 100 grams of fruc-

tose was followed by falls of 1 to 10 volumes per cent with simultaneous increases in the lactic acid content of the blood. Rose, Giragossintz, and Kirstein (11) injected 25 grams of fructose or glucose in 125 cc. of water into the small intestine and determined the lactic acid in the portal, the hepatic and the femoral veins and femoral artery. There was an increase of 50 to 100 per cent in the lactic acid content of the portal vein after injection of fructose but no significant change after glucose. The lactic acid disappeared in the liver as the hepatic vein showed but slight increase in lactic acid content. Wierzuchowski and Laniewski (14) determined the lactic acid content of the blood and urine in fasting dogs before and after the intravenous injection of 2 grams per kilo. per hour of solutions of glucose, fructose, and galactose for 2 hours. All three sugars brought about an increase in the lactic acid content of the blood with fructose producing the greatest increment. The rises of lactic acid in the blood were accompanied by an increased lactic acid elimination in the urine and by reciprocal changes in the  $\text{CO}_2$ -combining power of the blood.

Most of the studies reported in the literature indicate that an increase in the lactic acid content of the body results from the introduction of fructose. However, many of the investigations were made by the intravenous injection of the sugar solution and it is not improbable that the character of the metabolism of a substance differs according to the route by which it is introduced. In a number of studies, the doses were large and overloading may have led to abnormal metabolism. In some studies, the ingestion of glucose also brought about similar changes in the blood reaction and lactic acid content although not always to the same degree as was brought about by the introduction of fructose.

This publication gives the results of another method of attack on the problem of the metabolism of glucose and of fructose, that is, a comparison of the total respiratory quotients with the composition of alveolar air at intervals after the ingestion of the sugars.

#### *The Respiratory Exchange and Alveolar Air as Affected by the Ingestion of 25 grams of Glucose or of Fructose*

*Method of study.*—The subjects and procedures used were the same as those described in the preceding paper. The respiratory exchange was measured in 4 15-minute periods with the subjects in the post-absorptive condition. There was then a rest period of about 15 minutes, when the subject drank a solution of 25 grams of glucose or fructose in 200 cc. of water at  $37^\circ\text{C}.$ , and immediately inserted the mouthpiece of the respiration



apparatus. Measurements were begun at once and continued uninterruptedly for 10 successive 15-minute periods. The amount of glucose or fructose taken had been found in previous studies to cause definite rises in the respiratory quotient and metabolism (Carpenter and Fox, 2, 3) with a return to nearly pre-ingestion values in two and one-half hours. The alveolar air samples were collected once or twice each 15-minute period during the post-absorptive portions of the experiments and at the first and eighth minute of each period after the ingestion of the sugars.

*The Respiratory Quotient before and after the Ingestion of 25 grams  
of Glucose or Fructose*

The experiments reported here were made primarily for the purpose of comparing the respiratory quotients as affected by the ingestion of 25 grams of glucose or fructose with the alveolar air collected at intervals. The respiratory quotients for J. C. are plotted in Figures 1 and 2. The quotients in the 4 post-absorptive or base-line periods show the same tendency to low values in the first periods with slightly higher values in the succeeding periods, as in the series in the preceding paper (see p. 41). After the ingestion of glucose, the quotients reached a maximum in the ninth 15-minute period, and were sustained at a high level until the tenth to eleventh period of the experiment. They subsequently fell but even at the end of two and one-half hours after the sugar ingestion they did not reach the post-absorptive quotient in all the experiments.

The effect of the fructose was similar to that which has been found in previous investigations, the maximum occurring usually in the third quarter-hour after ingestion, with a return to nearly normal at the end of two and one-half hours. Attention is called to the high pre-ingestion quotients of May 12, accompanied by the much greater maximum height of the quotient after fructose as compared with the course of the quotients in the other three experiments with the same subject.

The respiratory quotients of A. F. S. before and after the ingestion of glucose and fructose are shown in Figures 3 and 4. The first preliminary post-absorptive quotients were lower than usually found in a normal subject as there are several quotients below 0.75. In the eighth period of the glucose experiments (third after ingestion) there was a rise in the respiratory quotient, but this was not uniform.

In the first two periods of the experiments after the ingestion of fructose, there was but little effect on the respiratory quotient due to the ingestion of sugar, in the third period there was a marked rise, and in the fourth

period occurred the maximum respiratory quotient after the ingestion of fructose. The period of maximum rise was slightly later with this subject than with J. C. and the uniformity in the rise in the quotient was not so great as with J. C.

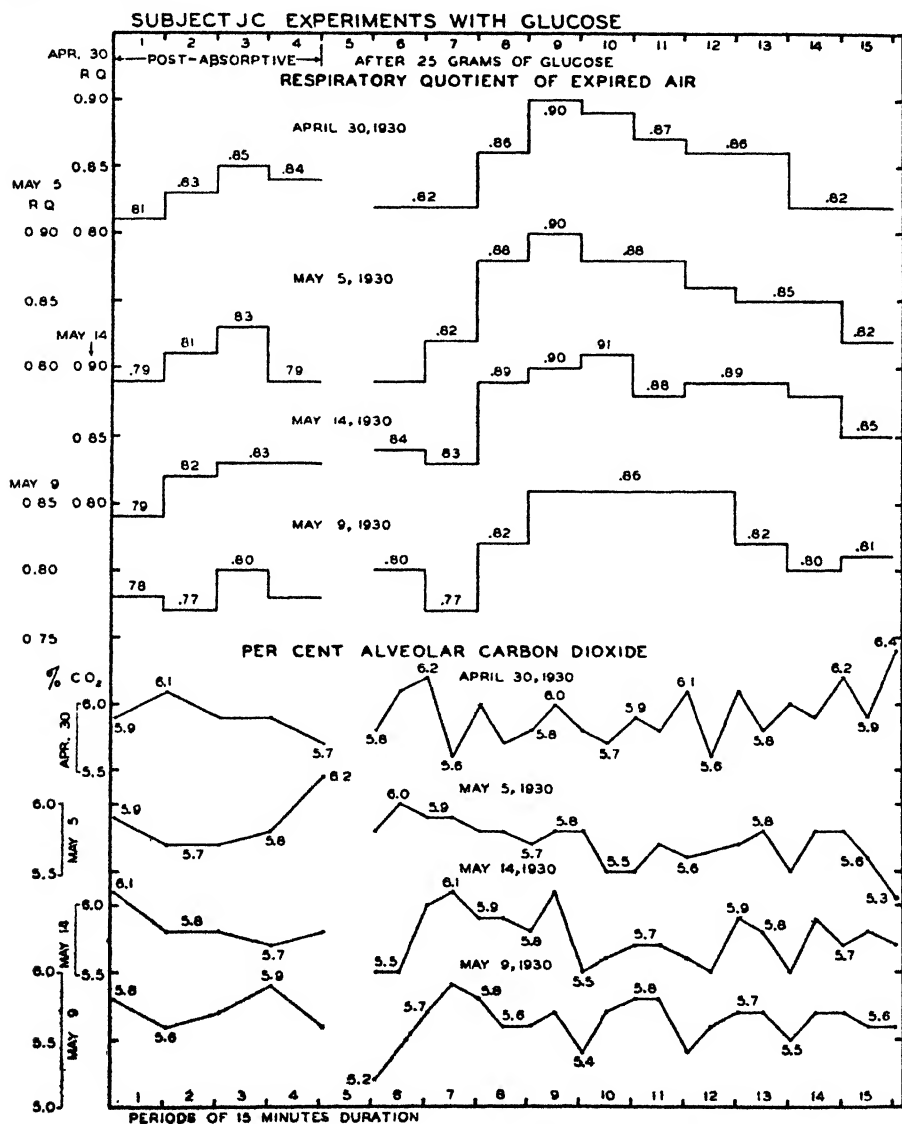


FIG. 1. Respiratory quotient of expired air and percentage alveolar carbon dioxide in individual experiments with J. C. before and after the ingestion of 25 grams of glucose.

*Percentage of Alveolar Carbon Dioxide as Affected by the Ingestion of 25 grams of Glucose or Fructose*

The individual determinations of percentage of alveolar carbon dioxide for J. C. before and after the ingestion of 25 grams of glucose are plotted

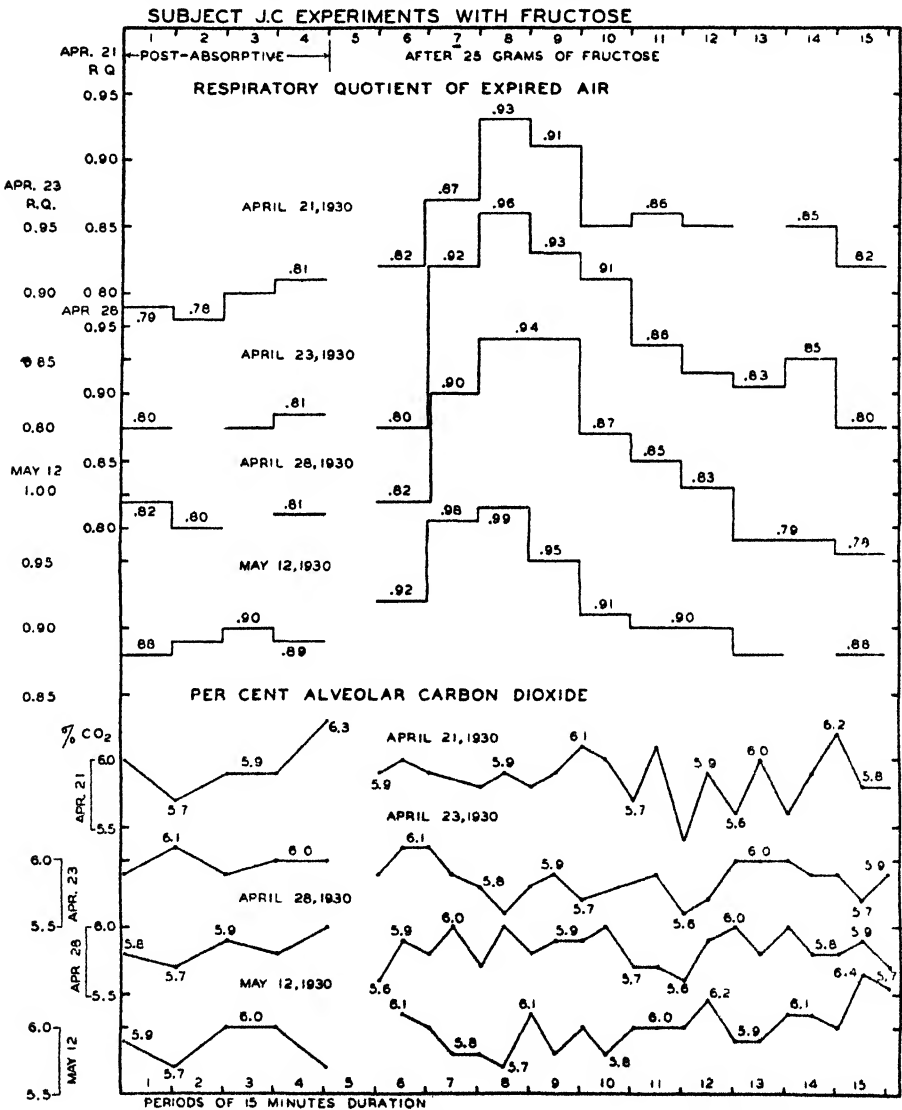


FIG. 2. The respiratory quotient of the expired air and the percentage alveolar carbon dioxide in experiments with J. C. before and after the ingestion of 25 grams of fructose.

in Figure 1 and for the experiments with fructose in Figure 2. The values in the four 15-minute periods before ingestion of the sugars in both groups varied from 5.6 to 6.3 in the extremes, although the majority of the values were within narrower limits, 5.7 to 6.0 per cent, and there is no tendency

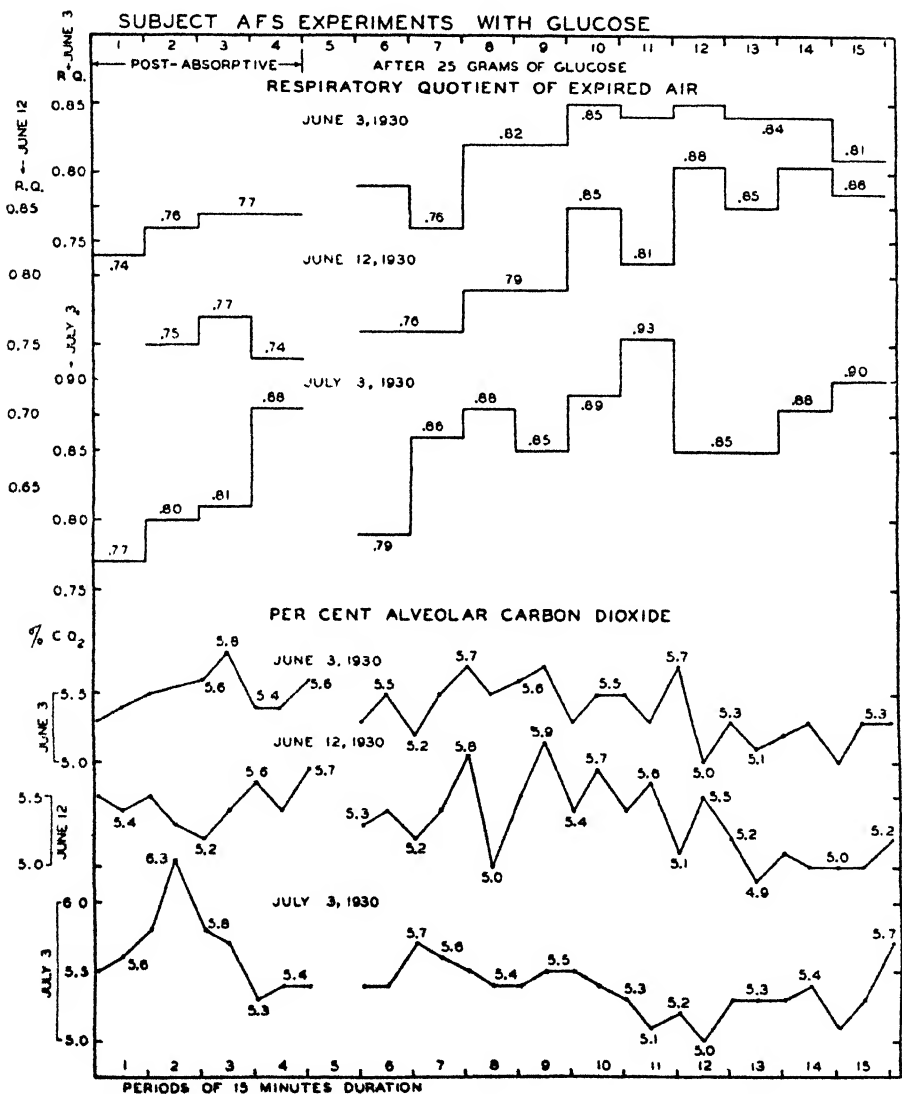


FIG. 3. Respiratory quotient of expired air and percentage of alveolar carbon dioxide in the individual experiments with A. F. S. before and after the ingestion of 25 grams of glucose.

to a rise or a fall. In three out of four experiments with glucose there was no definite change as the result of the ingestion of the sugar.

There was but little significant change in the alveolar carbon dioxide throughout the two and three-quarter hours after fructose was given, al-

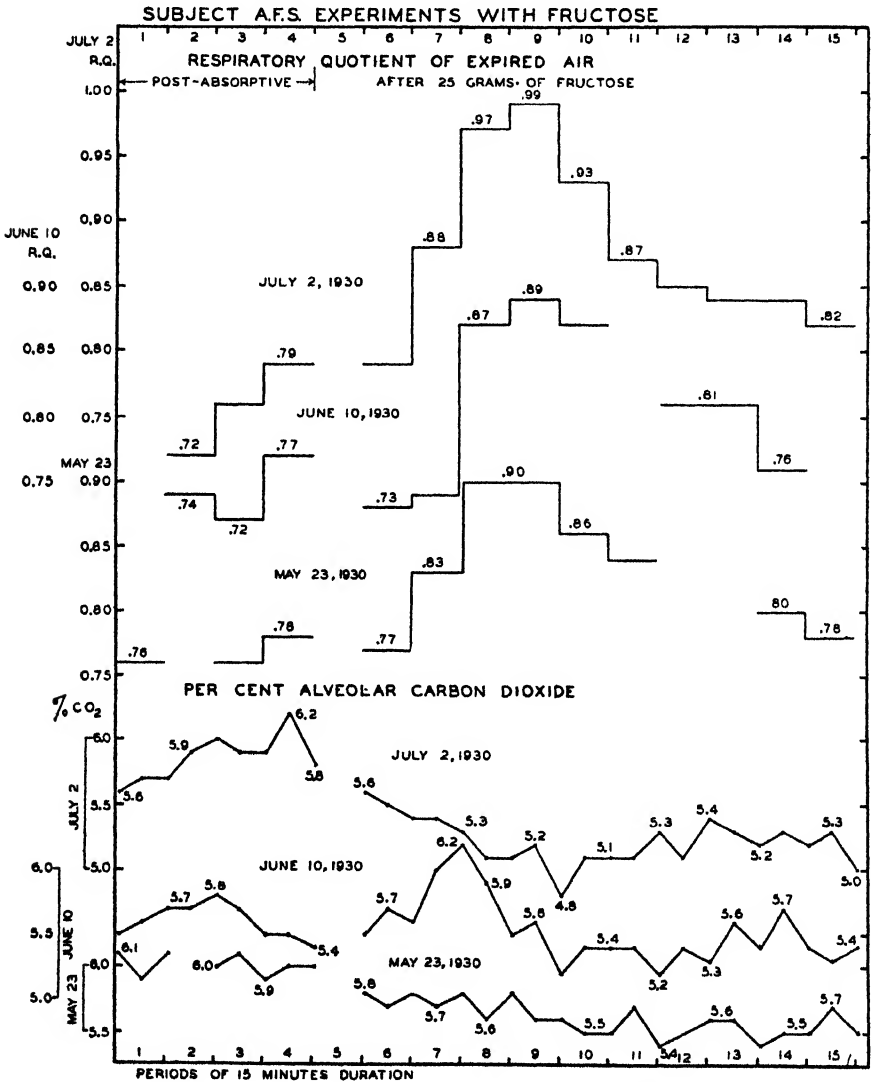


FIG. 4. The respiratory quotient of the expired air and the percentage alveolar carbon dioxide in experiments with A. F. S. before and after the ingestion of 25 grams of fructose.

though there were fluctuations from one determination to another, with occasionally marked variations. Curiously, in the twelfth period or one and three-quarter hours after ingestion, there was a particularly low value ranging from 5.4 to 5.6 in three of the experiments out of four, but this was the only point of time during the whole series in which there was a noticeable deviation from the normal level in practically the majority of the experiments. This was not the period in which the respiratory quotient began to fall off after the peak had been reached. The peak was several periods before this, so that the rate of fall of the quotient was no more marked in this particular period than in any of the others. After this period the average alveolar carbon dioxide was practically the same as the pre-ingestion level, with a range from 5.9 to 6.0.

With this subject the rise in the respiratory quotient and subsequent fall after the ingestion of fructose were in no way due to an alteration in the alveolar carbon dioxide, and a corollary to this would be that there was no change in the blood bicarbonate or total carbon dioxide.

The percentages of alveolar carbon dioxide for A. F. S. before and after the ingestion of 25 grams of glucose or fructose are plotted in Figures 3 and 4 respectively. The values before ingestion show a range of 5.2 to 6.3, with the majority between 5.4 and 5.7. The level is thus lower than with J. C. and lower than with the same subject in the preceding study, in which all the experiments were made with the subject in a post-absorptive state. In general, with A. F. S. there was a fall in the alveolar carbon dioxide about one and one-half hours after the ingestion of glucose, which is nearly the same period at which the maximum respiratory quotients were found.

Like the experiments with no dose in the preceding paper and the experiments with glucose there was a noticeable tendency for the alveolar carbon dioxide to fall off during the course of the experiments after the ingestion of fructose, and after the fourth period after ingestion, the carbon dioxide of the alveolar air was low. This is one-quarter hour later than the marked rise in quotient which took place with this subject after fructose.

#### *The Average Respiratory Quotient of Expired Air*

In Figures 5 and 6 are plotted the average respiratory quotients of J. C. for the two groups of experiments with glucose and fructose for the base-line hour and for 10 15-minute periods succeeding the ingestion of the sugars. In the experiments with glucose there was a variation in the quotient of expired air of 0.040 in the 4 base-line periods. After the ingestion

of glucose there was no apparent effect for one half-hour. Then there was a marked rise, so that within the next half-hour the peak was reached. Beginning one and one-half hours after the ingestion there was a gradual fall, so that one hour later the quotient was 0.015 above the average base-line respiratory quotient. The maximum rise from the base line was 0.08

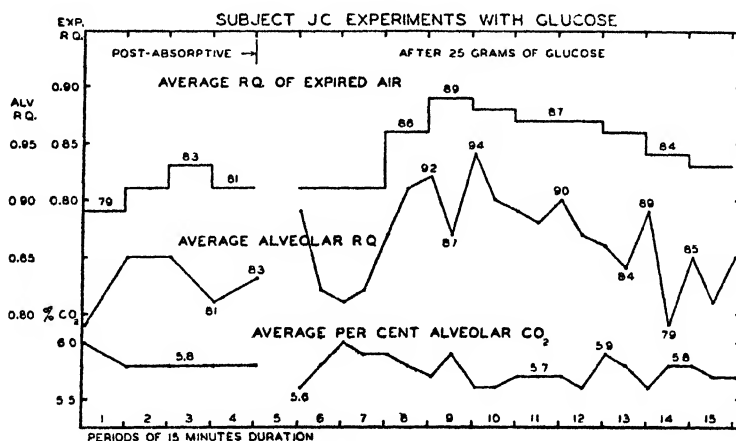


FIG. 5. Average respiratory quotient of expired air, average alveolar respiratory quotient, and average percentage alveolar carbon dioxide in experiments with J. C. before and after the ingestion of 25 grams of glucose.

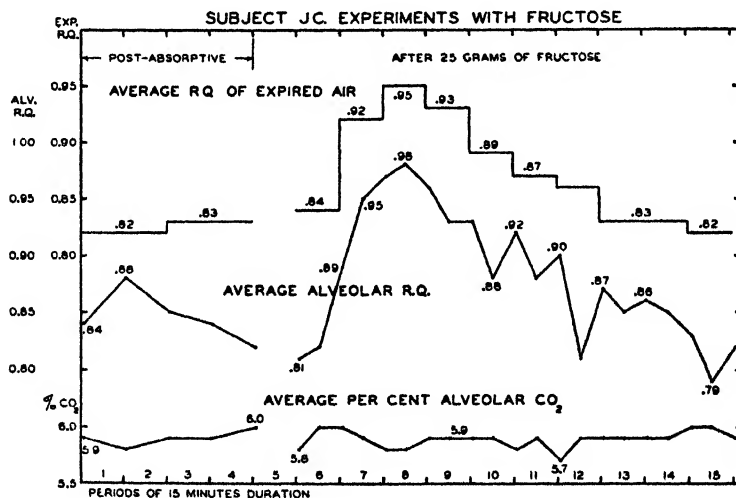


FIG. 6. Average respiratory quotient of expired air, average alveolar respiratory quotient, and average percentage of alveolar carbon dioxide in experiments with J. C. before and after the ingestion of 25 grams of fructose.

and there was a marked increase above the base-line level for one and one-half hours. In the experiments with fructose, the base-line or pre-ingestion periods of J. C. had a nearly uniform average respiratory quotient of 0.82 and 0.83. After the ingestion of the sugar, there was a sharp rise to a maximum of 0.95 in the eighth period of the group or the third quarter-hour after ingestion. From there on until the end of the experiments the average respiratory quotients fell to 0.82, the pre-ingestion level.

The average respiratory quotients of the expired air of A. F. S. for the two groups of experiments with glucose and fructose are shown in Figures 7 and 8. In the group with glucose there was a steady rise of 0.040 in the

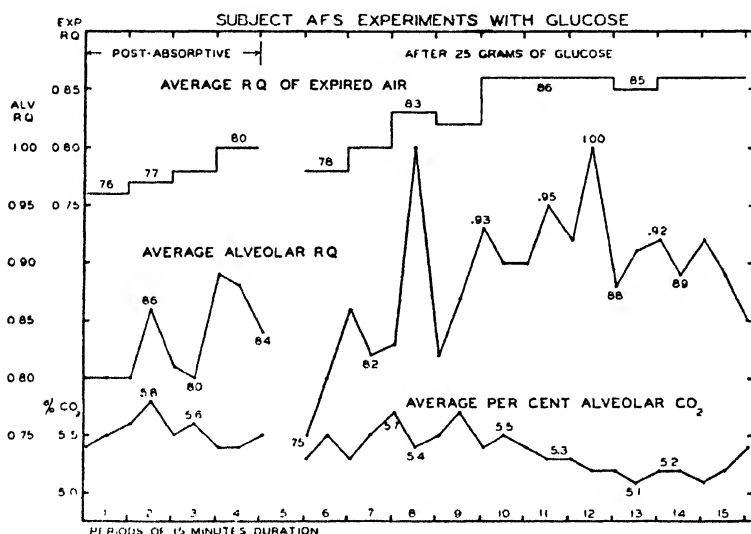


FIG. 7. Average respiratory quotient of expired air, average alveolar respiratory quotient, and average percentage of alveolar carbon dioxide in experiments with A. F. S. before and after the ingestion of 25 grams of glucose.

4 base-line periods before ingestion. Immediately after the ingestion there was but a slight change until the third quarter-hour in which the rise was 0.05 above the average base line. During the next half hour the quotient rose so that it was 0.08 above the base line. This higher level lasted for practically the rest of the experiment, that is, during a total of one and one-half hours. The rise in the respiratory quotient after the ingestion of glucose with both subjects, therefore, was about of the same order, although the initial level is 0.035 lower with A. F. S. than with J. C. The difference between the two subjects was that with A. F. S. there was not



a fall shortly after the peak was reached as there was with J. C. The course of the quotient after the ingestion of glucose with J. C. was more nearly like that in previous studies than was that of A. F. S.

In the group of experiments with fructose, the average respiratory quotients showed a rise in the 3 pre-ingestion periods from 0.74 to 0.78. After the ingestion of 25 grams of the sugar there was a rise from 0.77 to 0.92 in the ninth period or the fourth quarter-hour after ingestion. From there on until the end of the experiment, there was a fall to 0.80, a value 0.04 above

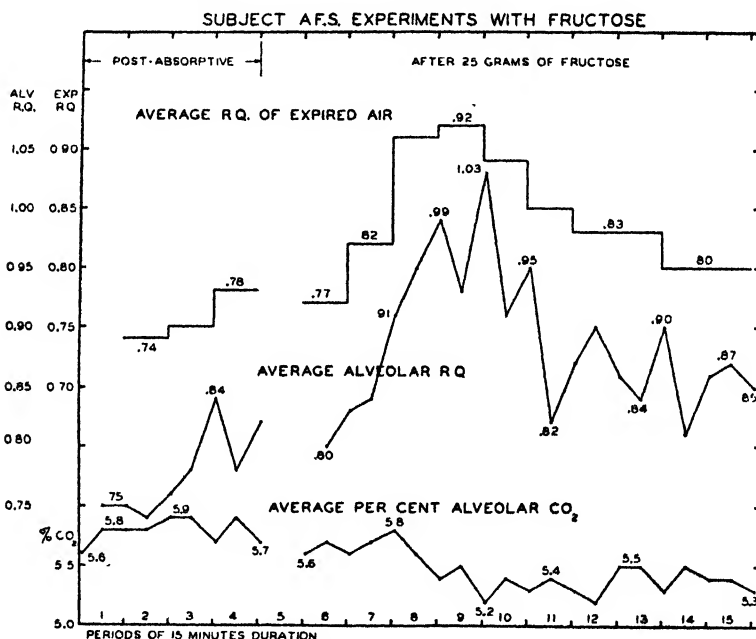


FIG. 8. Average respiratory quotient of expired air, average alveolar respiratory quotient, and average percentage of alveolar carbon dioxide in experiments with A. F. S. before and after the ingestion of 25 grams of fructose.

the average pre-ingestion level of 0.76. Thus there was a greater rise in the respiratory quotient after fructose with A. F. S. than with J. C. but part of this rise, as explained later, was due to a fall of the alveolar carbon dioxide with an accompanying over-ventilation or extra elimination of carbon dioxide over that produced as the result of the ingestion of the fructose.

#### *Average Alveolar Respiratory Quotient*

The average alveolar respiratory quotient of J. C. is shown in Figures 5 and 6 for the groups of experiments with glucose and fructose respectively.

After the ingestion of glucose there were marked variations from one period to another, but the smoothed curve which could be drawn from the determinations is somewhat of the same shape as the respiratory quotient of the expired air. Its peak, however, is somewhat higher, averaging about 0.92 and, in general, the alveolar respiratory quotient will average slightly higher all through the experiments than the respiratory quotient of the expired air.

After the ingestion of fructose there was a sharp rise in the first three periods to a maximum of 0.98 in the third period after ingestion, which is 0.03 higher than the respiratory quotient of the expired air in the same period. From there on there was an irregular fall to nearly the same level as the respiratory quotients of the expired air at the end of the experiments. In general, a smoothed curve of the alveolar respiratory quotients follows the course of the respiratory quotients of the expired air, although at a generally higher level than that of the expired air.

The average alveolar respiratory quotients for A. F. S. are plotted in Figures 7 and 8 for the experiments with glucose and fructose respectively. They were very irregular for the preliminary hour. The average alveolar respiratory quotient after the ingestion of glucose was extraordinarily irregular, but a smoothed curve taken from the points which show a rise in the respiratory quotient, beginning at the third one-quarter hour (period 7) after ingestion would correspond nearly to the changes in the respiratory quotient of the expired air.

The average alveolar respiratory quotients of A. F. S. after the ingestion of fructose were much more irregular than those of J. C. but as a whole the alveolar respiratory quotients of A. F. S. showed the same tendency to a parallelism with the respiratory quotients of the expired air as J. C.

In general one can conclude from the respiratory quotient of the alveolar air that its course, when taken as a whole, corresponds somewhat to the course of the respiratory quotient of the expired air, but that with both subjects the alveolar quotient is slightly higher.

#### *Average Percentage Alveolar Carbon Dioxide*

The average percentage alveolar carbon dioxide in the two groups of experiments with J. C. is plotted in Figures 5 and 6. During the preliminary base-line hour the maximum difference was 0.20 per cent. At the beginning of the period after the ingestion of glucose, the alveolar carbon dioxide on the average is lower than during the base-line hour, but during the next 15 minutes rises to a level that, on the average, is about the same as that

during the base-line hour. There was but slight alteration or change in direction of the alveolar carbon dioxide percentage. As judged by the percentage of carbon dioxide in the alveolar air, there is no indication that the respiratory quotient is other than a metabolic one, that is, that the rise and fall in the respiratory quotient represent an increase and a decrease in the proportion of carbohydrates catabolized.

The course of the alveolar carbon dioxide in the experiments with fructose was for the most part regular throughout the whole series of periods. The range was 0.30 in the 21 points after ingestion of fructose. There is no definite indication of a change in the course of the alveolar carbon dioxide after the ingestion of fructose which is greater than the changes in the pre-ingestion periods.

The average percentage alveolar carbon dioxide in the two groups of experiments with A. F. S. is plotted in Figures 7 and 8. The average percentage during the base-line hour was nearly uniform. Beginning with the 5th 15-minute period after the ingestion of glucose (period 10), the percentages, on the average, were lower by about 0.40 and tended to fall off gradually. Thus the last part of the change in respiratory quotient of expired air due to the ingestion of glucose with this subject is accompanied by a change in the alveolar carbon dioxide percentage. Whether this is an effect due to glucose or whether it is the result of the natural course of the alveolar air of this subject during the morning hours cannot be stated.

In the third period after ingestion of fructose (eighth of the experiments) there was a definite drop in the average alveolar carbon dioxide and during the remainder of the experiments it averaged between 5.5 and 5.2 per cent, thus definitely lower than the range of the pre-ingestion level (5.6–5.9). The ingestion of 25 grams of fructose with A. F. S. was therefore accompanied by a significant lowering of the alveolar carbon dioxide from the period of the maximum respiratory quotient until the end of the experiments. Therefore not all of the rise in the respiratory quotient of the expired air with A. F. S. as the result of ingestion of fructose can be ascribed to a metabolism unaccompanied by a change in level of the alveolar carbon dioxide.

So far as J. C. is concerned, it can be stated definitely that the effect of the ingestion of fructose was not to change in any way the alveolar carbon dioxide, and one would be warranted by this standard alone to draw the conclusion that the respiratory quotient after the ingestion of fructose with this subject represented the metabolic changes only and not the effect in part or wholly of the shift in equilibrium of the blood. On the contrary,

with A. F. S. one would be justified in drawing a conclusion that the effect of fructose was to cause the alveolar carbon dioxide to fall and, therefore, there had been an elimination of more carbon dioxide than was produced as the result of the ingestion of fructose. This subject showed, however, the same sort of results when experiments were carried out the same length of time and no dose was given. Consequently it is a question whether a fall of the alveolar carbon dioxide is the result of the ingestion of fructose or whether it is a normal characteristic of this subject regardless of what material is ingested. Therefore, the respiratory quotient studied with a subject such as this cannot be of the same significance as quotients obtained from subjects who, in post-absorptive condition with no dose, show a uniform alveolar carbon dioxide from hour to hour throughout the period of four to five hours.

*Relationship between Alveolar Respiratory Quotients  
and Alveolar Carbon Dioxide*

The relationship between the alveolar respiratory quotient and the alveolar carbon dioxide is plotted for J. C. and A. F. S. in Figure 9. The alveolar respiratory quotients of J. C., for the most part, ranged between 0.80 and 0.92, but the alveolar carbon dioxide for the same limits of respiratory quotients ranged between only 5.5 and 5.9 per cent. The extremes are a low respiratory quotient of 0.765 with an alveolar carbon dioxide of 6.18 per cent and a respiratory quotient of 0.97 for a carbon dioxide value of 5.2. Thus the relationship between the alveolar respiratory quotients and the alveolar carbon dioxide is most marked at the extreme limits of the ranges of both values, that is, the higher the alveolar carbon dioxide, the lower the alveolar respiratory quotient. The alveolar respiratory quotients which vary from 0.80 to 0.92 are probably due to the changes in the character of the metabolism and these correspond to the changes in the respiratory quotient of the expired air.

With A. F. S. on June 3, there is more of a relationship between the respiratory quotient of the alveolar air and the carbon dioxide of the alveolar air than in the experiments with J. C. The quotients ranged, in the majority, between 0.75 and 0.91, and the alveolar carbon dioxide ranged between 5.15 and 5.7 per cent. The highest respiratory quotients of 1.02, 0.956, and 0.940 correspond to low carbon dioxide of the alveolar air, namely, 5.00 and 5.08 and 5.03. These extremes are probably abnormal, both with respect to the respiratory quotient and the alveolar air. In other words, one would scarcely be so sure of drawing the conclusion that either

all the alveolar respiratory quotients or the respiratory quotients of the expired air represented metabolic changes. The results with A. F. S. are for the most part similar to those obtained with J. C. but had a wider range in both alveolar carbon dioxide and alveolar respiratory quotient.

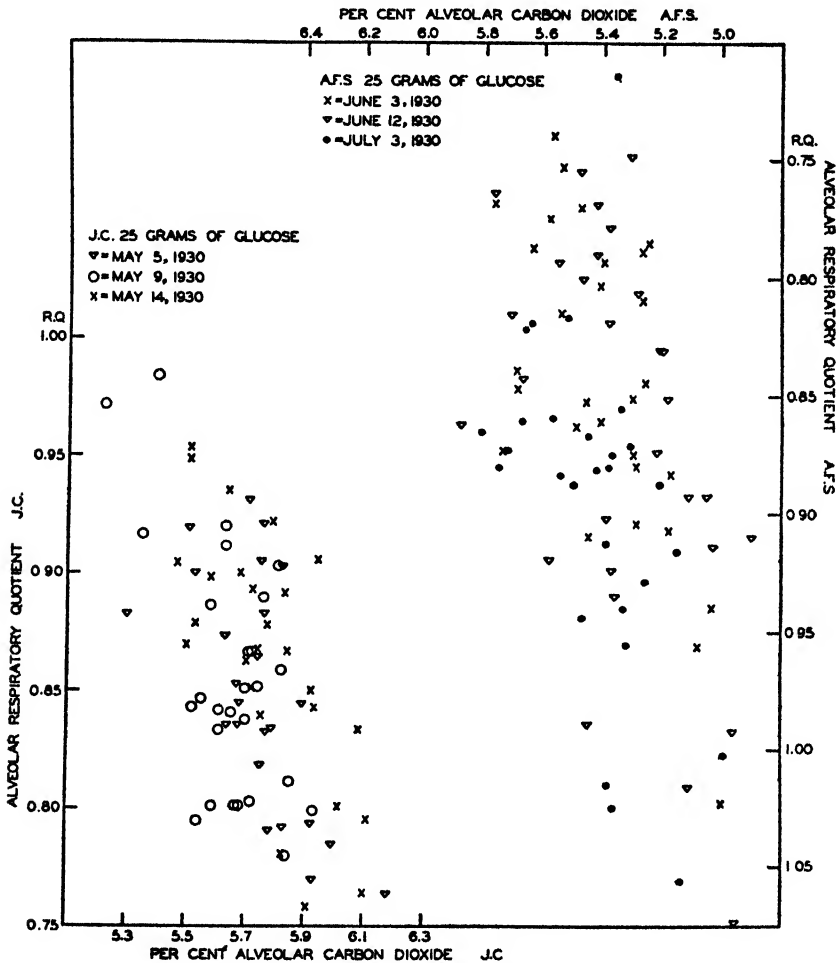


FIG. 9. Diagram showing relationship between alveolar respiratory quotient and percentage alveolar carbon dioxide in experiments with J. C. and A. F. S. before and after ingestion of 25 grams of glucose. The experiments with A. F. S. are plotted in an inverse manner to those of J. C.

The alveolar respiratory quotients as well as the quotients of the expired air are not so significant with A. F. S. as indications of the metabolic changes as are those of J. C. because of the shifts in the percentages of the alveolar carbon dioxide during the course of the observations. To be

of significance the alveolar carbon dioxide should remain practically constant throughout the periods.

A plotting of the relationships between the alveolar respiratory quotient

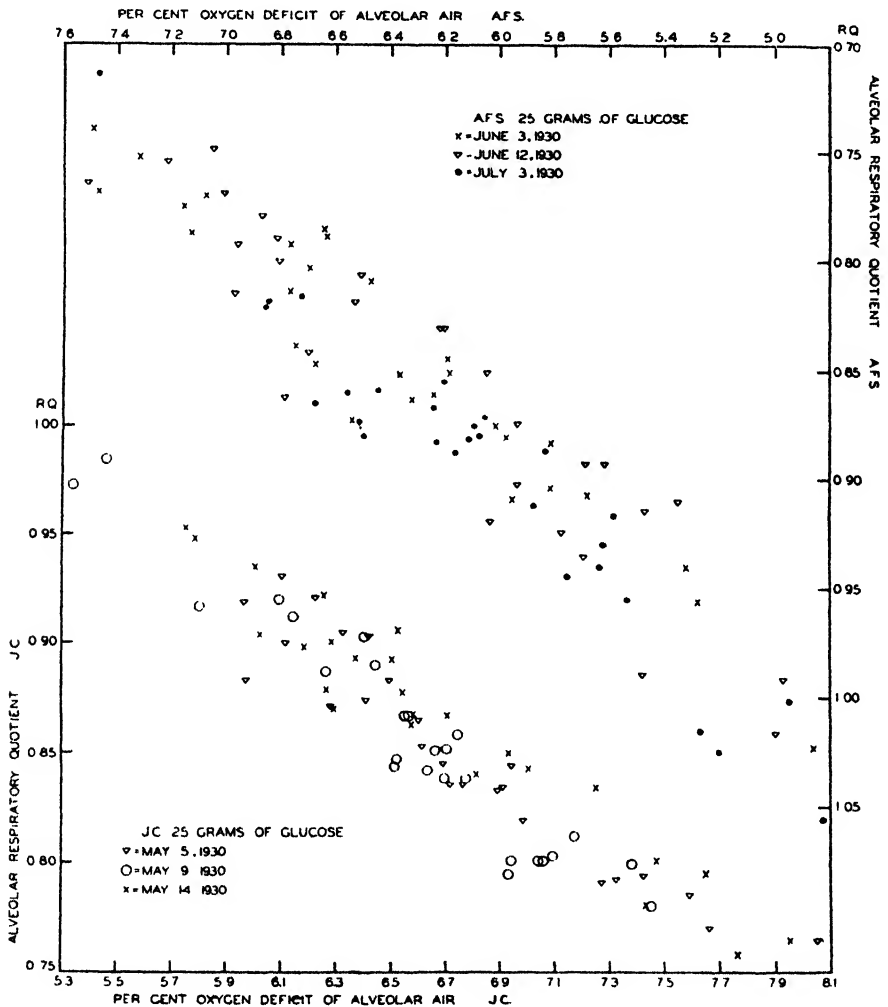


FIG. 10. Diagram showing the relationship between the alveolar respiratory quotient and the alveolar oxygen deficit in experiments with J. C. and A. F. S. before and after the ingestion of 25 grams of glucose. The experiments with A. F. S. are plotted in an inverse manner to those of J. C.

and the alveolar carbon dioxide in the experiments with fructose shows essentially the same results as the experiments with glucose.

*Relationships between Alveolar Respiratory Quotients and  
Percentage Alveolar Oxygen Deficits*

The relationships between the alveolar respiratory quotient and the alveolar oxygen deficit in experiments with J. C. and A. F. S. before and after the ingestion of 25 grams of glucose are plotted in Figure 10.

With J. C. on May 5, the alveolar respiratory quotients varied from 0.76 to 0.93 and the oxygen deficits from 6.0 to 8.05 per cent, with a distinct inverse relationship between the two factors. There is a slightly greater scatter in the values at the high respiratory quotients and low oxygen values than in the lower quotient values. This is in part due to the generally lower percentages in the alveolar carbon dioxide. All three experiments show that the alveolar respiratory quotient varied inversely with the alveolar oxygen deficit.

With A. F. S. there was a greater range in both the alveolar respiratory quotients and the alveolar oxygen deficits. In general there is the same relationship between the alveolar respiratory quotient and the alveolar oxygen deficit with A. F. S. as with J. C., that is, the higher the quotient, the lower the oxygen deficit. The extremely low oxygen deficits with the extremely high quotients are in part due to a more dilute alveolar air which was characteristic of this subject after the first one and one-half hours following the ingestion of glucose.

When the values for the experiments with fructose were plotted, the same relationships were found between the alveolar respiratory quotients and alveolar oxygen deficits as in the groups of experiments with glucose. In both groups of experiments, the results indicate that the majority of the alveolar respiratory quotients were metabolic respiratory quotients and not the results of disturbances in the breathing or of changes in the reaction of the blood. This is particularly true of J. C. with whom there were relatively few abnormally high or low alveolar carbon dioxide percentages.

*Correlation Coefficients between the Alveolar Respiratory Quotient  
and the Composition of the Alveolar Air*

In Table I are given the arithmetical means of the alveolar respiratory quotient, alveolar carbon dioxide and alveolar oxygen deficit with the average variations from the means and the standard deviations, and the correlation coefficients between the alveolar respiratory quotient and the alveolar carbon dioxide and the alveolar oxygen for J. C. and A. F. S. in the experiments with 25 grams of glucose or 25 grams of fructose.

TABLE I  
CORRELATION COEFFICIENTS BETWEEN THE ALVEOLAR RESPIRATORY QUOTIENTS AND THE ALVEOLAR CARBON DIOXIDE AND OXYGEN DEFICIT  
IN EXPERIMENTS WITH GLUCOSE AND FRUCTOSE

Subject and date	Number of determinations	Alveolar R.Q.		Alveolar CO <sub>2</sub>		Alveolar O <sub>2</sub> deficit		Correlation coefficients		
		Average	S.D.	Average	S.D.	Average	S.D.	Alveolar R.Q. and alveolar CO <sub>2</sub>	Alveolar R.Q. and alveolar O <sub>2</sub> deficit	
									r	P.E. <sub>r</sub>
1930				%		%				
25 grams of glucose										
J. C.										
May 5	25	0.850 ± 0.041	0.048	5.74 ± 0.12	0.17	6.75 ± 0.45	0.55	-0.629 ± 0.082	-0.949 ± 0.013	
May 9	26	0.857 ± 0.042	0.053	5.65 ± 0.12	0.16	6.59 ± 0.38	0.51	-0.588 ± 0.087	-0.961 ± 0.010	
May 14	26	0.865 ± 0.041	0.052	5.77 ± 0.15	0.19	6.67 ± 0.48	0.59	-0.700 ± 0.067	-0.964 ± 0.009	
A. F. S.										
June 3	29	0.845 ± 0.052	0.065	5.40 ± 0.17	0.21	6.40 ± 0.52	0.65	-0.621 ± 0.077	-0.956 ± 0.011	
June 12	30	0.856 ± 0.074	0.094	5.34 ± 0.20	0.24	6.30 ± 0.72	0.91	-0.486 ± 0.094	-0.954 ± 0.011	
July 3	30	0.902 ± 0.057	0.076	5.45 ± 0.18	0.25	6.06 ± 0.52	0.65	-0.405 ± 0.103	-0.925 ± 0.018	
25 grams of fructose										
J. C.										
April 21	25	0.862 ± 0.052	0.060	5.89 ± 0.15	0.20	6.84 ± 0.54	0.62	-0.529 ± 0.097	-0.941 ± 0.016	
April 23	24	0.865 ± 0.043	0.058	5.87 ± 0.12	0.16	6.79 ± 0.42	0.54	-0.532 ± 0.099	-0.955 ± 0.012	
April 28	26	0.861 ± 0.054	0.062	5.84 ± 0.10	0.13	6.79 ± 0.46	0.53	-0.258 ± 0.123	-0.960 ± 0.010	
May 12	25	0.905 ± 0.048	0.066	5.97 ± 0.14	0.18	6.61 ± 0.51	0.72	-0.825 ± 0.043	-0.979 ± 0.006	
A. F. S.										
May 23	30	0.793 ± 0.047	0.057	5.75 ± 0.18	0.20	7.26 ± 0.61	0.72	-0.611 ± 0.077	-0.948 ± 0.013	
June 10	30	0.838 ± 0.090	0.106	5.53 ± 0.19	0.23	6.71 ± 0.91	1.04	-0.531 ± 0.088	-0.976 ± 0.006	
July 2	30	0.884 ± 0.092	0.124	5.42 ± 0.27	0.33	6.27 ± 0.96	1.27	-0.623 ± 0.075	-0.956 ± 0.011	



The correlation coefficients between the alveolar respiratory quotient and the alveolar carbon dioxide in the experiments with glucose indicate a tendency for the alveolar carbon dioxide to vary inversely as the alveolar respiratory quotients. This is logical because the alveolar carbon dioxide will depend to a large extent on the correctness of the sampling. If the sample is drawn at the time of over-ventilation the alveolar air will be diluted as well as extra carbon dioxide washed out, and the respiratory quotient will be too high. Conversely, if the alveolar ventilation is inadequate, the alveolar carbon dioxide will be too high and the alveolar respiratory quotient too low.

The correlation between the alveolar respiratory quotient and the alveolar oxygen deficit was high in this group of experiments with both subjects. The alveolar respiratory quotient varied inversely as the oxygen deficit. In spite of the variations in alveolar carbon dioxide, the oxygen deficit varies inversely much more closely with the alveolar respiratory quotient than does the alveolar carbon dioxide. In fact, the correlation coefficients between the alveolar oxygen deficit and the alveolar respiratory quotient are higher than the correlation coefficients between the oxygen deficit of the expired air and the respiratory quotient of the expired air (unpublished). The alveolar air is not directly influenced by either the dead space or the respiration rate, as is the composition of expired air.

The correlation coefficients of the alveolar carbon dioxide and the alveolar respiratory quotient in the experiments with fructose were all negative, thus indicating that the carbon dioxide varied inversely as the quotient. However, only half of them are above 0.6, and the coefficients of the others hardly exceed 6 times the probable error. The correlation between the alveolar carbon dioxide and alveolar respiratory quotient is not significant. The correlation coefficients of the alveolar respiratory quotient and the alveolar oxygen deficit were negative and of a very high order, that is, the respiratory quotient varied inversely as the oxygen deficit of the alveolar air, and this applies without exception to both subjects.

#### *Comparison of Alveolar Air during the Base-line Hour and the Period of Maximum Respiratory Quotient after the Sugars*

Figures 1 to 4 show the general course of the respiratory quotients of expired air before and after the ingestion of glucose and of fructose and the course of the percentage of carbon dioxide in the alveolar air, and in Figures 5 to 8 are shown the averages of the alveolar carbon dioxide, the alveolar respiratory quotient, and the respiratory quotient of the expired

air. In some experiments there were marked variations and a closer analysis of the possible changes in the respiratory quotient due to the ingestion of the sugars is desirable. For this purpose Table II has been prepared. In

TABLE II.

COMPARISON OF ALVEOLAR CARBON DIOXIDE AND RESPIRATORY QUOTIENT DURING BASE-LINE HOUR WITH THOSE DURING PERIODS OF MAXIMUM RESPIRATORY QUOTIENT AFTER INGESTION OF 25 GRAMS OF GLUCOSE AND OF FRUCTOSE

Subject and date	Base-line hour			Periods of maximum R.Q. after sugars		
	(a) Average R.Q.	(b) Average alveolar CO <sub>2</sub> %	(c) Average alveolar R.Q.	(d) Average R.Q.	(e) Average alveolar CO <sub>2</sub> %	(f) Average alveolar R.Q.
1930						
25 grams of glucose						
J.C.						
April 30	0.840	5.87	—	0.885	5.85	—
May 5	0.805	5.86	0.805	0.885	5.65	0.895
May 9	0.785	5.71	0.835	0.860	5.63	0.900
May 14	0.830	5.77	0.860	0.895	5.80	0.895
Average	0.815	5.80	0.830	0.880	5.73	0.895
A. F. S.						
June 3	0.770	5.53	0.785	0.845	5.36	0.890
June 12	0.750	5.43	0.815	0.870	5.11	0.935
July 3	0.795	5.72	0.890	0.890	5.25	0.970
Average	0.770	5.56	0.830	0.870	5.24	0.930
25 grams of fructose						
J. C.						
April 21	0.795	5.94	0.820	0.905	5.90	0.910
April 23	0.800	5.96	0.830	0.935	5.81	0.935
April 28	0.810	5.86	0.840	0.925	5.86	0.935
May 12	0.890	5.84	0.900	0.975	5.87	0.970
Average	0.825	5.90	0.850	0.935	5.86	0.940
A. F. S.						
May 23	0.755	6.00	0.740	0.890	5.63	0.885
June 10	0.745	5.61	0.785	0.875	5.61	0.925
July 2	0.755	5.90	0.810	0.960	5.11	1.045
Average	0.750	5.84	0.780	0.910	5.45	0.950

column (a) is given the average base-line respiratory quotient for each experiment which is made up of periods 2 to 4 for the most part. The column (b) shows the average per cent of carbon dioxide in the alveolar air for the same periods. In column (c) is given the average alveolar respiratory quotient for the corresponding periods. In the right hand portion of the table there is given the average [column (d)] of the respiratory quotients of expired air of the three periods which were the highest after ingestion of the sugar. In nearly all cases with glucose these began in the ninth or tenth period. On May 9 the average included the four periods 9 to 12 and on May 14, periods 8 to 10, that is, in practically all cases the maximum respiratory quotient occurred at about the same interval of time after the ingestion of sugar. In the experiments with fructose the highest quotients were in the periods 7 to 9 with J. C. and 8 to 10 with A. F. S. In column (e) is given the average alveolar carbon dioxide for the periods selected for the highest respiratory quotient. In column (f) is given the average alveolar respiratory quotient for these periods. With J. C. the average respiratory quotient in the base-line hour in the experiments with glucose varies from 0.78 to 0.84, but the alveolar carbon dioxide has a range of only 0.16 per cent for the 4 experiments. In two cases the average alveolar respiratory quotient was higher than the average respiratory quotient of the expired air, and in these two cases the average alveolar carbon dioxide was slightly lower than the other two of the four. The difference between the respiratory quotient of expired air and the alveolar respiratory quotient is not large, viz., 0.015 on the average. In two of the experiments the average alveolar carbon dioxide is practically the same in the periods of maximum respiratory quotient as that during the base-line hour. In the other two these percentages are 0.08 and 0.21 lower than those in the base-line hour, and in these two (May 5 and May 9) the average alveolar respiratory quotient after glucose is slightly higher than the average respiratory quotient of the expired air. The average alveolar carbon dioxide percentage after the sugar is lower by 0.07 than the average during the base-line hour. After glucose the difference between the average respiratory quotient of the expired air and the average respiratory quotient of the alveolar air is 0.015.

With A. F. S. the average respiratory quotient in the base-line hour in the experiments with glucose as well as the percentage of carbon dioxide in the alveolar air was materially lower than with J. C. and the difference between the respiratory quotient of the expired air and that of the alveolar air was more marked, being 0.06 on the average. The average value of the respiratory quotient of the periods of maximum height was 0.10 higher

than that of the base-line hour, a greater difference than was obtained with J. C. Similarly, the average percentage of carbon dioxide of the alveolar air for the periods of maximum respiratory quotient was lower by 0.32 than that of the base-line hour. There was also, on the average, a much higher alveolar respiratory quotient during the periods of maximum respiratory quotient than was obtained with J. C., and the difference between the respiratory quotient of the expired air and that of the alveolar air was 0.06. Therefore, with J. C. it is apparent that the average maximum respiratory quotient after the ingestion of glucose is of significance because there was but little change in the percentage of carbon dioxide in the alveolar air, and the respiratory quotients of the alveolar air agreed in the main with those of the expired air. On the contrary, with A. F. S. part of the rises in the respiratory quotient after the ingestion of glucose must have been due to a fall of the alveolar carbon dioxide, so that there were combined the effects of the increase in the metabolism of carbohydrates due to the utilization of glucose and of a fall of the carbon dioxide content of the body, with the result that the respiratory quotient was raised more in the case of A. F. S. than with J. C.

Additional evidence may be obtained from a consideration of the course of these factors for the same periods of time in the group of experiments in which no dose was given (see preceding article). In Table III are grouped for the experiments with no dose the values for the periods of time corresponding to those with the ingestion of glucose, particularly with reference to the maximum respiratory quotient after the ingestion. With J. C. there is a satisfactory agreement in the respiratory quotient for the two groups of periods as well as for the carbon dioxide of the alveolar air. This is true not only with respect to the average, but also with respect to the individual experiments so far as the alveolar air is concerned. There are only two experiments in which the respiratory quotient was determined for the alveolar air, but the differences between the average alveolar respiratory quotient and that of the expired air are not wide. On the contrary, with A. F. S. there is a marked difference between the average respiratory quotient of the base-line hour and that of the periods corresponding to the maximum respiratory quotients in the glucose experiments. There was an increase of 0.04 in the respiratory quotient and a decrease of 0.45 in the alveolar carbon dioxide percentage, thus indicating an approximate increase in the respiratory quotient of 0.01 for each fall in the alveolar carbon dioxide percentage of 0.10. If we apply a correction of this character to the values obtained in Table II after the ingestion of glucose, we shall

TABLE III  
AVERAGE ALVEOLAR CARBON DIOXIDE AND RESPIRATORY QUOTIENT FOR THE SAME PERIODS IN NO-DOSE EXPERIMENTS AS THOSE COMPARED FOR THE MAXIMUM RESPIRATORY QUOTIENTS IN THE GLUCOSE AND FRUCTOSE EXPERIMENTS

Subject and date	Preliminary hour			No-dose periods*			No-dose periods**		
	(a) R.Q.	(b) Alveolar CO <sub>2</sub> %	(c) Alveolar R.Q.	(d) R.Q.	(e) Alveolar CO <sub>2</sub> %	(f) Alveolar R.Q.	(g) R.Q.	(h) Alveolar CO <sub>2</sub> %	(i) Alveolar R.Q.
1930									
J.C.									
April 14	0.795	5.92	—	0.795	—	—	0.780	—	—
April 16	0.825	6.08	—	0.800	5.92	—	0.805	5.92	—
April 18	0.830	6.30	—	0.795	6.22	—	0.800	6.24	—
May 7	0.765	5.96	0.790	0.790	6.09	0.765	0.790	6.01	0.785
May 16	0.820	5.86	0.830	0.800	6.02	0.785	0.810	5.89	0.825
Average	0.805	6.02	0.810	0.795	6.06	0.775	0.800	6.02	0.805
A. F. S.									
May 27	0.770	5.73	0.800	0.775	5.68	0.785	0.775	5.73	0.795
June 26	0.810	5.98	0.860	0.860	5.42	0.895	0.850	5.51	0.895
June 28	0.775	6.07	0.800	0.835	5.33	0.880	0.830	5.46	0.855
Average	0.785	5.93	0.820	0.825	5.48	0.855	0.820	5.57	0.850

\* Corresponding to periods of highest respiratory quotients in the experiments with glucose.

\*\* Corresponding to periods of highest respiratory quotients in the experiments with fructose.

have a decrease of the average respiratory quotient after the ingestion of glucose of 0.03 corresponding to a difference of 0.32 between the average alveolar carbon dioxide in the base-line hour and that in the periods of maximum respiratory quotient. This decreases the difference between the average respiratory quotient of the expired air during the base-line hour and the periods of maximum respiratory quotient to 0.070, which is practically the same as the difference between the averages found with J. C. Therefore, when the respiratory quotient of the maximum periods is corrected for the possible change due to a fall of alveolar air based upon experiments in which no sugar was given, we find that the rise in respiratory quotient due to glucose alone is practically the same in both cases. This method of analysis of the results with A. F. S. makes useful experiments on him which would otherwise be of doubtful significance. The results with A. F. S. (an untrained subject) thus supplement and confirm the results found with J. C., a trained subject.

In the experiments with fructose (Table II) the averages of the alveolar CO<sub>2</sub> for the base-line hour with J. C. show a narrow range, 0.12 per cent, from day to day, with an average of 5.90 for the four days. The average respiratory quotient of the alveolar air was slightly higher than that of the expired air.

The average maximum respiratory quotient of J. C. after the ingestion of fructose was 0.935, and this rise of 0.11 over the base-line hour occurred in the periods 7 to 9 after the ingestion of fructose. The differences in three of the four experiments were close to the average difference. The average carbon dioxide of the alveolar air in the periods of maximum R. Q. was remarkably close to that of the average alveolar carbon dioxide in the base-line hour, both with regard to the individual experiments and the averages of the four. The agreement indicates that the increase in respiratory quotient was a metabolic one, that is, due to the transformation of carbohydrates. The average respiratory quotient of the alveolar air was also close to that of the expired air. Thus with J. C. the evidence is very consistent with regard to the influence of the ingestion of fructose on the respiratory exchange in indicating that the rise in the respiratory quotient was due to the metabolic changes which took place after the ingestion of fructose.

With A. F. S., the average respiratory quotient in the base-line hour was lower than with J. C., although the alveolar carbon dioxide of A. F. S. was very close to that of J. C. There is not a marked difference between the average respiratory quotient of the alveolar air and that of the expired air during the base-line hour. The difference between the respiratory quotient

of the base-line hour and the maximum respiratory quotient after fructose was 0.160, which is a larger increase than that of J. C. The average alveolar carbon dioxide in two of the three experiments was lower than that of the base-line hour and the average respiratory quotient of the alveolar air was somewhat higher than that of the expired air.

In Table III are given the average respiratory quotients for the periods in the post-absorptive experiments given in a preceding article (see p. 37) corresponding to those of the maximum respiratory quotients after the ingestion of fructose. With J. C. there is no difference between the base-line hour and the periods of maximum respiratory quotient, in the respiratory quotient of expired air, carbon dioxide of alveolar air, and alveolar respiratory quotient. With A. F. S., on the contrary, there is a higher respiratory quotient in the periods corresponding to the maximum with fructose and also a lower alveolar carbon dioxide per cent as well as a high alveolar respiratory quotient. If we correct the increase in respiratory quotient after the ingestion of fructose with A. F. S. by 0.01 corresponding to each 0.10 per cent decrease in the alveolar carbon dioxide, we shall have the same increase in the respiratory quotient of the expired air with A. F. S. as with J. C., namely, 0.12 with A. F. S. as compared with 0.11 with J. C. Therefore the greater portion of the rise in the respiratory quotient with A. F. S. was due to the metabolic process after the ingestion of fructose and but a small proportion due to the fall of the alveolar carbon dioxide. The maximum respiratory quotient after the ingestion of 25 grams of fructose is 0.05 greater than the maximum rise in the respiratory quotient after the ingestion of glucose and there is, therefore, a marked difference between the effects of fructose and glucose upon the respiratory exchange. Although the difference in the maximum rise in the quotient may not appear to be large enough to be considered of much significance, it should be noted that the quantities of sugar ingested were small (only 25 grams) in comparison with the amounts used with human subjects, and the differences are always found consistently when these two sugars are used.

#### SUMMARY

The respiratory exchange and composition of the alveolar air were determined with two human subjects on 3 and 4 days for four post-absorptive 15-minute periods and for ten 15-minute periods after the ingestion of 25 grams of glucose and of fructose.

There was no change in the alveolar carbon dioxide with the trained subject accompanying the rises in the respiratory quotient after the inges-

tion of the sugars, but with an untrained subject there was a marked fall in the alveolar carbon dioxide during the periods of maximum rise in the respiratory quotient.

With the trained subject there was not a definite relationship between the alveolar carbon dioxide and the alveolar respiratory quotient in the experiments with fructose, but in the experiments with glucose and in the two groups of experiments with the untrained subject there was a tendency to a negative correlation between the alveolar carbon dioxide and the alveolar respiratory quotient. There was a marked negative correlation between the alveolar oxygen deficit and the alveolar respiratory quotient in both groups of experiments with both subjects. The alveolar respiratory quotients tended to run parallel with the respiratory quotients of the expired air.

There was a maximum rise in the respiratory quotient of 0.065 in the experiments with glucose with one subject and 0.10 with the other subject. When the respiratory quotient after the ingestion of glucose with the untrained subject was corrected for the rise in respiratory quotient corresponding to the falls in the alveolar carbon dioxide equivalent to those which took place after the ingestion of glucose, the net increase in the respiratory quotient was then the same as with the trained subject.

The average maximum rise in the respiratory quotient of the expired air with the trained subject in the experiments with fructose was 0.11 and was accompanied by nearly the same rise in the alveolar respiratory quotient with no significant change in the alveolar carbon dioxide. The average maximum rise in the respiratory quotient of the expired air with the untrained subject was 0.16, accompanied by a rise of 0.17 in the alveolar respiratory quotient and a decrease in the alveolar carbon dioxide of 0.4 per cent. When the respiratory quotient of the expired air is corrected for the apparent rise due to a fall of alveolar carbon dioxide found in previous post-absorptive experiments with this subject, the increase in the respiratory quotient of the expired air becomes 0.12, thus practically the same as with the trained subject.

Therefore a comparison of the course of the alveolar air and the respiratory quotient in the post-absorptive condition with the changes taking place after the ingestion of sugars gives a basis for the calculation of the true effect of the ingested sugars on the respiratory exchange.

If the constancy of the alveolar air is to be taken as an indication of true metabolic respiratory quotients, as has been frequently suggested in the literature, then the net rise in respiratory quotients in these experiments



as the result of ingestion of fructose must be regarded as a result of the metabolism of fructose without the formation of organic acids in its transformation in sufficient quantities to affect the carbon dioxide tension of the alveolar air.

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## A COMPARISON OF APRICOTS AND THEIR CAROTENE EQUIVALENT AS SOURCES OF VITAMIN A

By

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THE recent experimental work on vitamin A and carotene points definitely to a correlation between these two substances. While the evidence is against their being identical, there is ample reason to assume that carotene is a source of vitamin A and may perhaps be considered its pro-vitamin. The work to date shows that the pro-vitamin A is synthesized by plants and is found in the highest concentrations in the green and yellow leaves, seeds and roots. The flowers, fruits, and vegetables which have been shown to contain an abundance of pro-vitamin A are rich in the carotenoid pigments and in many cases chlorophyll is also present. In any case, carotene appears to be closely associated with the formation of vitamin A or a pro-vitamin A in plants and with its utilization by animals. The question of the quantitative equivalence of the two substances cannot be said to have been established so far. It seemed worth while then to attack this question by simultaneous chemical and biological analyses of a typical food containing carotene and acting as source of vitamin A in measurable amounts. The fruit chosen was the apricot, partly because its vitamin A value had already been studied rather exhaustively in this laboratory and partly because of its exceptional activity as source of vitamin A and its concurrent richness in carotenoid pigments.

The plan of experiments here reported involved:

1. The quantitative estimation of the carotene contents of the apricot samples used, viz. fresh, frozen, unsulfured sun-dried, and sulfured sun-dried specimens.
2. The biological testing of these same samples for vitamin A activity.
3. The biological testing of the vitamin A value of crude and purified carotene fed in amounts equivalent to some of those found in the apricot samples and used in the vitamin A testing of the fruit.

There should emerge from these determinations some suggestions as to the truth of the supposition of the dependence of the vitamin value of the fruit samples upon their carotene content. Likewise, the interrelation of

amount of destruction of both carotene and vitamin A simultaneously in the dried samples of the apricots might offer additional evidence as to the equivalence of these two substances.

Royal apricots, having a deep yellow flesh, were chosen for the test, and the same variety lot, and crop were used in the three forms tested. All the fruit samples were taken from the same orchard. The fresh fruit was preserved by freezing and storage at  $-17^{\circ}$  after having been packed in small cans which were then evacuated and refilled with nitrogen twice. Two sun-dried samples were used, one of which was sulfured for 4 hours. The fresh fruit, S1, contained 79.4 per cent moisture, the sulfured sun-dried sample, S2, had a moisture content of 17.5 and contained 480 parts of sulfur dioxide per million. The unsulfured sun-dried sample, S3, contained 14.7 per cent water.<sup>1</sup>

The carotene contents of the fruits were colorimetrically determined on the petroleum ether and ether soluble fractions. The usual biological test with rats was used for vitamin A efficiency of the fruits and of the carotene which was obtained from the British Drug Houses, Ltd. and purified by recrystallization from petroleum ether.

The carotene of the samples was extracted by two methods, by pyridine as suggested by Smith and Spoehr (1) and developed by L. L. W. Smith in this laboratory (2) and by acetone and ether as described by Schertz (3). The pyridine was used in several portions, about 15 times the weight of the sample (which was usually 10 grams) being needed for complete extraction of the carotenoid pigments. The pyridine was removed with normal sulfuric acid and the pigment taken up in petroleum ether, dried with anhydrous sodium sulfate, made up to volume and the color read against the carotene standard of Sprague (4).

The acetone-ether extraction was carried out as modified by Schertz (3). The acetone and flavones were washed out with water and finally with one per cent sodium carbonate solution. The ethyl ether extract was evaporated at a temperature below  $50^{\circ}$  in a current of carbon dioxide. The residue was shaken up with petroleum ether and methyl alcohol, the latter taking up any xanthophyll which was present. But in the case of apricots xanthophyll appears to be absent since the methyl alcohol washings were colorless. The petroleum ether layer was then washed with water, dried with anhydrous sodium sulfate, made up to volume and read against the Sprague standard.

<sup>1</sup> The fruit was prepared and this analysis furnished by P. F. Nichols of the Fruit Products Division, College of Agriculture, University of California.

TABLE I  
COMPARATIVE EXTRACTIONS OF CAROTENE BY PYRIDINE AND ACETONE-ETHER

Sample	Number of determinations	Carotene per gram of fruit				Carotene per gm. of fruit solids
		Acetone-ether extractions	Pyridine extract of acetone-ether extracted residues	Acetone-ether extract and pyridine extract of residues	Pyridine extractions	
		mg.	mg.	mg.	mg.	mg.
S1. Fresh frozen apricots, 1930 crop	4	—	—	—	0.021	0.102
	5	0.018	—	—	—	0.088
Fresh apricots, 1931 crop	5	0.023	—	—	—	—
	3	—	—	—	0.024	0.115
S2. Sulfured sun-dried apricots	4	0.041	0.014	0.056	—	0.066
	3	—	—	—	0.055	0.066
	4	0.042	—	—	—	0.050
S3. Unsulfured, sun-dried apricots	4	—	—	—	0.051	0.060
	4	0.040	—	—	—	0.047
	6	0.039	0.011	0.050	—	0.059

The pigment values found by these 2 methods as shown in Table I do not agree closely, the pyridine extracts being in all cases except that of the fresh fruit richer in carotene than the acetone-ether extracts. It was at first suspected that some loss occurred during the evaporation of the ethyl ether extract in the latter process. Since xanthophylls appear to be negligible in amount in apricots, the methyl alcohol separation and ether evaporation were omitted in a new trial of the Schertz separation and values were obtained identical with those found when these steps were not omitted. The pulp residues from the acetone-ether extractions were then further extracted with pyridine by the method described and when the resulting solutions were read colorimetrically against the standard were found to contain enough pigment to account for the difference between the original pyridine and acetone-ether extracts. It is thus evident that pyridine removes pigments, particularly from the dried fruit samples, which the acetone and ether had not extracted. The nature of this pigment is doubtful. It may be either carotene or lycopersicin since Smith and

Smith (5) found the latter pigment to be more readily soluble in pyridine than in acetone and ether.

The fresh frozen apricots which were used for the vitamin tests were found to have a maximum of 0.102 mg. carotene per gram of fruit solids as compared with 0.066 and 0.060 for the sulfured and unsulfured samples, indicating losses of 36 and 41 per cent of the carotenoid values during the drying process. Interest attaches to the corresponding losses in vitamin A potency of these dried fruits.

TABLE II  
THE VITAMIN A ACTIVITY OF FRESH AND DRIED APRICOTS

Sample	Daily dose		Number of rats	Body weights		Total gain	Period	Weekly gains
		Equivalent in fresh fruit		Initial	Final			
	mg.	mg.		gms.	gms.	gms.	days	gms.
Fresh frozen apricots, S1	0.0	0	21	79	75		20	-1.3
	15.0	15	12	68	83	15 ± 3	35	3.0
	25.0	25	10	75	105	30 ± 3	46	4.6
	30.0	30	19	62	101	39 ± 1	52	5.2
	40.0	40	7	54	108	54 ± 5	54	7.8
	50.0	50	5	71	140	69 ± 4	56	8.6
Sulfured sun-dried apricots, S2	7.5	30	10	66	86	20 ± 2	38	3.6
	22.5	90	13	80	129	49 ± 3	54	6.3
	30.0	120	16	103	154	51 ± 2	53	6.7
	37.5	150	9	75	138	63 ± 2	56	7.9
Unsulfured sun-dried apricots, S3	7.5	31	12	68	89	21 ± 2	40	3.6
	22.5	93	16	109	137	28 ± 3	49	4.0
	30.0	124	20	106	148	42 ± 2	54	5.4

The vitamin tests were made by the usual biological method, both loss of weight and appearance of ophthalmia being used as criteria of vitamin A depletion. The doses of apricot used were chosen in accord with previous findings (6) in this laboratory on similar samples. As shown in Table II, the 15 mg. daily dose of the fresh fruit supports an increase of 3 grams body weight per week, the usual Sherman standard for the vitamin A unit. Twice the equivalent of this amount must be fed as either sulfured or unsulfured sun-dried apricot in order to obtain about the same amount of growth. A similar relation appears to be shown by the effects of the larger doses, 25 and 30 mg. of the fresh fruit giving about the same rate of in-

crease as 90 to 120 mg. (fresh equivalent) of the sulfured dried fruit or 93 to 124 mg. of the unsulfured fruit. It is fair to say then that only 25 to 50 per cent of the vitamin A activity of the fresh fruit is retained in the dried specimens. The loss of vitamin cannot be stated so exactly as can the corresponding loss of carotene content, but it is apparent that these losses are of the same general magnitude, although the carotene destruction tends to be distinctly lower, 36 to 41 per cent as compared with 50 to 75 per cent loss of vitamin, possibly because of better absorption of carotene from the fresh fruit.

Crystalline carotene of melting point  $162^{\circ}$ – $164^{\circ}$  was obtained from the British Drug Houses, Ltd. and was fed in ethyl laurate<sup>2</sup> in doses of 0.001, 0.002, and 0.005 mg. daily. Excellent growth was obtained on the larger doses and at the rate of 4.3 grams per week even on the 0.001 mg. dosage. The carotene was then recrystallized from its filtered solution in boiling purified petroleum ether, (boiling point  $30^{\circ}$ – $60^{\circ}$ ) three times with rise in melting point to  $179^{\circ}$ – $180^{\circ}$ . A fourth recrystallization of part of the material raised the melting point to  $181^{\circ}$ . The crystals which corresponded closely with the description of the crystals obtained by Olcovich and Mattill (7) were dried in an atmosphere of carbon dioxide and kept in the dark. The ethyl laurate solution used for feeding was made up, in small, frequently renewed portions, from the third crystal crop. Doses of 0.001, 0.002 and 0.005 mg. daily of the purified carotene as indicated in Table III

TABLE III  
VITAMIN A ACTIVITY OF CAROTENE

Description of sample	Daily dose	Number of rats	Body weights		Total gain	Period	Weekly gains
			Initial	Final			
Crude carotene, m.pt. $162^{\circ}$ – $164^{\circ}$	mg.		gms.	gms.	gms.	days	gms.
	0.001	10	80	112	$32 \pm 7$	52	4.3
	0.002	10	79	135	$56 \pm 6$	53	7.4
	0.005	6	91	163	$72 \pm 4$	56	9.0
Crystalline carotene, m.pt. $179^{\circ}$ – $180^{\circ}$	0.001	4	118	171	$53 \pm 5$	54	6.9
	0.002	4	111	198	$87 \pm 6$	56	10.9
	0.005	4	105	209	$104 \pm 4$	56	13.0

produced distinctly better growth in vitamin A depleted rats than did the similar doses of the crude carotene. The vitamin activity appeared to be

<sup>2</sup> The ethyl laurate was kindly supplied by S. Lepkovsky of the Anatomical Laboratory of the University of California.

considerably increased by the purification, an outcome not unexpected if the crude substance contained oxidized or otherwise changed carotene products which were removed by the recrystallization. It is possible also that the original carotene contained a larger proportion of the less active alpha-carotene than that recrystallized from petroleum ether solution. The melting point of the alpha isomer is usually given as several degrees lower than that of the beta form. Kuhn and Lederer (8) found the solubility in n-hexane of the alpha isomer to be three times that of the beta-carotene and Kuhn and Brockman (9) as well as Karrer, Euler, Hellstrom, and Rydbom (10) report a slightly greater and more rapid response from beta than from alpha-carotene when used as source of vitamin A.

The growth of 4 rats at an average rate of 6.9 grams per week on 0.001 mg. carotene daily, about the same rate produced by twice this dosage of the crude carotene, represents perhaps the best results so far reported with carotene as source of vitamin A. Collison, Hume, Smedley-Maclean, and Smith (11) and Moore (12) were able to get some cures of vitamin A deficient animals with 0.002 mg. carotene but Green and Mellanby (13) found the minimum dose, melting point  $174^{\circ}$ , necessary for complete protection to be somewhat larger, 0.020 mg. Olcovich and Mattill (7) used 0.005 mg. as their lowest dose. Kuhn and Brockmann (9) failed to get growth in vitamin A deficient rats with doses of 0.0017 mg. of beta-carotene.

Our rats fed 0.005 and 0.002 mg. of the carotene, melting point  $180^{\circ}$ , were cured of their deficiency symptoms and all survived the experimental period of 56 days. Three out of four of those given 0.001 mg. survived the full period and grew satisfactorily but on autopsy were found to exhibit internal stigmata of vitamin A deficiency. These stigmata were chiefly crater-like tumors of the stomach and pus infections at the base of the tongue and in the ears and lungs.

The suggestion of Olcovich and Mattill (7) that total growth of rats induced by the administration of a given amount of carotene, as 0.005 mg., be used as criterion of comparative vitamin A value was considered in evaluating our biological tests for carotene. As seen in Table IV however the growth per 0.001 mg. carotene decreases as the total amount fed is increased above the minimum. Thus 0.001 mg. daily fed for 54 days produces weight increases of 0.98 grams per 0.001 mg. carotene but when twice or five times that amount is given the figure drops to 0.78 and 0.37. This is possibly due to the fact that as Sherman and Burtis (14) have stated, growth beyond the minimum of 3 grams weekly may be limited by other

TABLE IV  
RELATION OF CAROTENE INTAKE TO WEIGHT GAINS

Source of of Carotene	Daily dose		Number of rats	Total amount of carotene fed	Total weight gain	Feed- ing period	Gain per 0.001 mg. carotene
	of substance	of carotene					
Fresh apricot	mg.	mg.		mg.	gms.	days	gms.
	15.0	0.00030	12	0.010	15	35	1.50
	25.0	0.00052	10	0.024	30	46	1.24
	30.0	0.00063	19	0.033	39	52	1.18
	40.0	0.00084	7	0.045	54	54	1.20
	50.0	0.00105	5	0.059	69	56	1.17
Sulfured dried apricot	7.5	0.00041	10	0.015	20	38	1.33
	22.5	0.00123	13	0.066	49	54	0.74
	30.0	0.00165	17	0.087	50	53	0.57
	37.5	0.00206	9	0.115	63	56	0.55
Unsulfured dried apricot	7.5	0.00038	12	0.016	21	40	1.31
	22.5	0.00114	16	0.056	28	49	0.50
	30.0	0.00153	20	0.082	42	54	0.51
Crude carotene, m.pt., 162°-164°	0.001	0.001	10	0.052	32	52	0.61
	0.002	0.002	10	0.106	56	53	0.53
	0.005	0.005	6	0.280	72	56	0.26
Recrystallized carotene, m.pt., 179°-180°	0.001	0.001	4	0.054	53	54	0.98
	0.002	0.002	4	0.112	87	56	0.78
	0.005	0.005	4	0.280	104	56	0.37

factors besides vitamin A. In the lower ranges of dosage our figures correspond however very well with those found by Olcovich and Mattill. In doses of 0.0003 to 0.0008 mg. daily the maximum gain of 1.18 to 1.50 gms. per 0.001 mg. carotene was obtained on all our fruit samples; at 0.001 to 0.002 mg. daily the figures drop to 0.50 to 1.17 and at the 0.005 mg. level the growth is only 0.26 and 0.37 gms. per 0.001 mg. carotene. These 3 levels correspond roughly with intakes which produce weekly gains of 3 to 5 gms., 6 to 8 gms. and 9 to 13 gms. for the 8 weeks period.

In nearly all cases the unsulfured dried apricot yielded growth in proportion to carotene intake which was distinctly lower than the other sources of carotene used. This is in accord with our suspicion that certain colored derivatives of carotene or a similar compound present in the unsulfured samples are extracted by both methods used for carotene de-



termination and that our reported figures for the carotene content of these samples are consequently higher than they should be.

Such a comparison of the growth obtained on graded doses of a food in terms of its carotene content may have value in improving the assay for vitamin A. Further standardization of growth responses to varying carotene intakes is needed however.

The responses obtained at approximately the 0.001 mg. level have some interest in connection with the tentative adoption of this dosage of standard carotene as the international unit of vitamin A activity by the Permanent Commission on Biological Standardization of the League of Nations Health Organization (15). The growth response from this amount of carotene or from equivalent amounts of foods should probably be accepted as maximum only when administered at a given daily level and for a definite period. The rate of gain obtained by daily administration of 0.001 mg. carotene in the series here reported, 6.9 mg. per week, is greater than that recommended for the assay of vitamin A activity of foods by Sherman and Munsell (16) but quite close to that suggested by Javillier, Baude, and Levy-Lageunnesse (17), Dutcher, Honeywell and, Dahle (18), and used by Morgan and Field (6).

Thus 0.001 mg. carotene, melting point  $180^{\circ}$ , produced approximately the same response as did 0.002 mg. carotene, melting point  $162^{\circ}$ – $164^{\circ}$ , 40 to 50 mg. fresh apricots, or 22.5 to 30 mg. sulfured dried apricots. The comparative rates of growth secured by the three doses of crystalline carotene and by the most nearly equivalent carotene-containing apricot doses are shown in Chart 1. This dosage of the fresh fruit contained 0.0008 to 0.0010 mg. carotene according to the pyridine extraction figure (Table I) and the sulfured dried fruit 0.0012 to 0.0016 mg. carotene. The largest amount of unsulfured dried apricots, 30 mg., tried did not yield as much curative effect as did 0.001 mg. carotene, although the effect of the smaller doses was comparable with that of like amounts of the sulfured product.

The biological activity of the recrystallized carotene may thus be said to be approximately 25 to 50 per cent greater than that of the crude product. The yield of the purified crystals was considerably less than one-half the weight of the crude product and, as stated before, the proportion of the alpha and beta isomers present in the two preparations may well have been quite different. The biological value of the fresh and sulfured sundried apricots may be considered to be approximately 100 per cent of the expected value based on carotene determinations in these fruit samples. The carotene of apricots would seem therefore to be as nearly completely

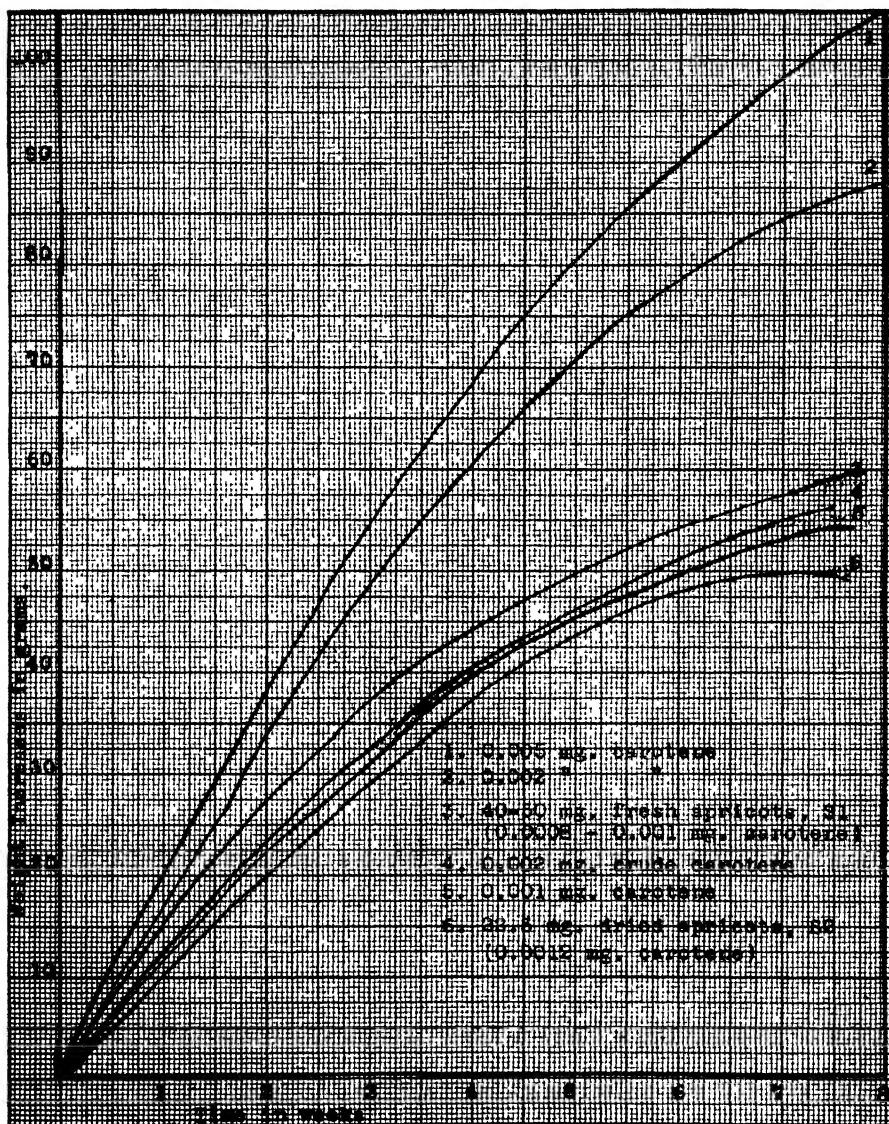


CHART 1. Growth curves of rats fed recrystallized and pure carotene and apricots as source of vitamin A.

convertible to vitamin A by the rat as the present methods of biological testing can determine. This may be tentatively interpreted to mean not only that all of the carotene of the fruit is available for transformation into vitamin A in the animal body, but also that no other constituent of the fruit is capable of such transformation. Whether the carotene of other plant products is capable of yielding such a large proportion of vitamin A can only be determined by further experiments.

The work of Ahmad (19) points to the importance of intestinal absorption in the determination of the efficiency with which food carotene is utilized as source of vitamin A in the animal body. Since completeness of carotene absorption may depend upon the amount and nature of such accompanying materials as fats, the quantitative determination of the carotene content of foods can not as yet be accepted as offering a dependable substitute for the biological testing of the food.

#### SUMMARY

1. The carotene content of fresh frozen, sulfured sun-dried and unsulfured sun-dried apricots of similar origin was determined by pyridine and by acetone-ether extraction followed by colorimetric estimation. The pyridine was found to extract the dried samples more completely and easily than did the acetone-ether. The loss of carotene in the dried fruit was 36 and 41 per cent.

2. The vitamin A activity of these fruit samples was determined biologically and the loss of vitamin in the dried specimens was found to be 50 to 75 per cent.

3. The vitamin A activity of crude carotene, melting point 162°–164°, was found to be less than that of the crystalline carotene, melting point 180°, made from it.

4. The apricots were seen to yield vitamin A in nearly the amounts indicated by their carotene content when compared with the vitamin value of similar doses of the crystalline carotene. The possible significance of this correlation is discussed.

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# THE COPPER, IRON, AND MANGANESE CONTENT OF FISH

By

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THE nutritional value of copper and manganese has recently attracted the attention of investigators. Hart and co-workers (1) were first to show the supplementing effect of copper to iron in hemoglobin formation. Later Titus, Cave, and Hughes (2), Goerner (3), and Myers and Beard (4) found manganese to possess a similar iron-supplementing property. Although the unique rôle of manganese in blood regeneration was not confirmed by Waddell and co-workers (5), Krause (6), Keil and Nelson (7), and Orent and McCollum (8), the experimental evidence, obtained by Mitchell and Miller (9), and Kemmer, Elvehjem, and Hart (10) indicates that manganese stimulates growth, and according to Orent and McCollum (8) manganese is essential for lactation and for the prevention of sterility.

The bearing of these investigations on practical nutrition has aroused an interest in the iron, copper, and manganese content of foods. Although valuable information as to the quantities of these elements contained in a wide variety of foods has been secured by Lindow, Elvehjem, and Peterson (11), Peterson and Elvehjem (12), Lindow and Peterson (13), Remington and Shiver (14), and Orent and McCollum (8), additional data on certain foods are still desirable. The purpose of this paper, therefore, is to report our results on the content of copper, iron, and manganese in twenty species of fish.

## EXPERIMENTAL

*Preparation of samples.* The fish used were bought in the local market and some were caught in Lake Champlain. Only fresh fish were used. After carefully skinning the fish, the edible muscle was stripped from the bones, placed in a prepared glazed porcelain evaporating dish and dried to constant weight at 100° in an electric oven.

Since Remington and Shiver (14), and others, have pointed out that there may be a variation between individuals within the same species, an effort was made to prepare representative samples by finely grinding

and thoroughly mixing together the dried edible muscles from a number of fish of the same kind. The dry, powdered material was finally stored in glass stoppered bottles until the analyses could be made.

*Methods of analysis.* The copper analyses were made by the method of Elvehjem and Lindow (15). Iron determinations were made by the method according to Stugart (16). Manganese determinations were made by the Skinner and Peterson (17) procedure.

## RESULTS

In Table I are given the percentage of moisture and the copper, iron, and manganese content, in mg. per kilo. of fresh moist material, for twenty species of fish.

TABLE I  
COPPER, IRON, AND MANGANESE CONTENT OF FISH MUSCLE

Name of Fish	Number used for sample	Moisture	Copper content of fresh muscle	Iron content of fresh muscle	Manganese content of fresh muscle
		per cent	mg. per kg.	mg. per kg.	mg. per kg.
Bass	3	77.0	2.1	4.2	0.26
Bullpout	10	80.0	2.4	9.4	0.24
Cod	5	79.3	3.8	3.4	0.13
Eel	4	55.5	1.7	5.1	0.27
Flounder	5	80.1	1.8	7.0	0.18
Haddock	4	79.1	2.3	4.8	0.15
Halibut	5	78.6	1.8	9.5	0.10
Herring	5	78.2	2.8	5.7	0.16
Herring (fresh water)	6	79.0	3.6	4.8	0.22
Ling	5	78.4	4.1	9.6	0.33
Mackerel	5	76.4	2.6	8.7	0.16
Muskellunge	2	78.8	2.5	6.2	0.23
Perch (yellow)	10	80.4	2.6	5.6	0.44
Pickrel	5	79.7	2.8	6.8	0.18
Pike	5	80.5	1.6	4.5	0.16
Salmon	4	75.0	2.0	8.6	0.14
Shad	5	73.6	2.2	4.8	0.18
Smelt (fresh water)	20	79.5	3.3	4.1	0.26
Sunfish (common)	15	80.3	1.4	3.4	0.29
Trout (lake)	4	78.0	3.1	7.2	0.31

*Copper.* The average copper content of both the salt water and fresh water fish was about the same. There were approximately 2.5 mg. per kilo.

fresh material in each case. This relationship is similar to that obtained by Lindow and Elvehjem and Peterson (11). They found that the average copper content for sixteen kinds of fish was 2.7 mg. per kilo. of fresh material. We found that the sunfish showed the lowest average copper content while ling was the highest, being 1.4 and 4.1 mg. per kilo. of fresh material respectively.

*Iron.* Our results in regard to the iron content of salt water and fresh water fish show that the former contain approximately 12 per cent more iron. The results of Peterson and Elvehjem (12) indicated that the salt water fish contained about 40 per cent more iron than was contained in the fresh water fish. This variation in our results is no doubt due to the fact that we analyzed more fresh water fish with dark-colored tissue. Our results are in agreement with those of Peterson and Elvehjem (12) in that the fish with dark colored tissue contained about 75 per cent more iron than those having light colored tissue. This was true in the case of both salt water and fresh water fish. Cod and sunfish contained the lowest amount of iron per kilo. of moist material, being 3.4 mg. in each case. Ling contained the greatest amount, the average amount being 9.6 mg. per kilo. of moist material.

*Manganese.* The manganese content was slightly greater in the fresh water fish than in the salt water type investigated. The various species of fish muscle contain about 0.1 mg. to 0.4 mg. per kilo. of moist material. This result is in agreement with the observations of Orent and McCollum (8), who state that fish muscle contains 0.1 mg. to 0.5 mg. of manganese per kilo. of moist material. Lindow and Peterson (13) found the muscle of certain species of fish to be manganese free. Skinner and Peterson (17) found that cod contained 6.3 mg. manganese per kilo. of dry material.

#### SUMMARY

The copper, iron, and manganese content of twenty species of fresh water and salt water fish have been determined. The content of copper in both the fresh and salt water fish averaged about 2.5 mg. per kilo. of moist material. Salt water fish contain about 12 per cent more iron than fresh water fish. Species of fish with dark colored tissue contain approximately 75 per cent more iron than the species with light colored tissue. Fish muscle contains small amounts of manganese.

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## THE VITAMIN A CONTENT OF YELLOW-TISSUED AND WHITE-TISSUED APPLES\*

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THE nutritional value of apples has not been investigated so extensively as that of some other fruits, although from the standpoint of production the apple stands toward the top of the list. Statistics (1) on the five-year-average car lot shipments of domestic fruits for 1925-26 to 1929-30 show that the car lot shipment of apples exceeds not only that of every other individual fruit, but exceeds as well the combined shipments of all citrus fruits.

Studies on apples and other plant foods have shown that the variety may be a significant factor in the establishment of vitamin values. Bracewell, Hoyle, and Zilva (2) found that the Bramley Seedling variety of apple was more potent in vitamin C than other varieties which they tested. Other studies of Bracewell *et al.* (3) on apple varieties showed that measurable differences existed among varieties in their vitamin C potency. Recent studies of Potter and Dickson (4) on the vitamin A value of different varieties of cherries indicate that certain varieties are more potent in this factor than others. Russell (5) found yellow dent corn to be 50 per cent more potent in vitamin A than a white-capped yellow dent variety. Steenbock and Boutwell (6) found yellow varieties of maize to be far superior to the white varieties as a source of vitamin A. Quinn and Cook (7) in quantitative studies of the vitamin A content of white and yellow yautia, showed the latter to be 10 times as rich in vitamin A as the white variety. Russell (5) has reviewed the more recent investigations concerning the correlation between yellow pigment and the presence of vitamin A.

In this experiment three varieties of apples were studied, two having yellow tissues and one having white tissues. Since yellow pigment has been associated with the presence of vitamin A in other products, it was thought of interest to investigate apples from this standpoint.

### MATERIALS AND METHODS

Thirty albino rats from six litters averaging approximately 41 gm. each in weight were used for the preliminary study. The rats were from stock fed

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the Sherman diet 13 consisting of 2/3 whole wheat, 1/3 whole milk powder, and 2 per cent sodium chloride based on the weight of wheat. The technic of Sherman and Munsell (8) for vitamin A studies was used essentially, and at 21 to 28 days of age the animals were placed on the experiment.

The vitamin A-free basal diet consisted of the following:

	Per cent
Cornstarch, irradiated	67
Casein, vitamin A-free	18
Powdered dry yeast	10
Osborne and Mendel salt mixture	4
Sodium chloride	1
	<hr/> 100

In the preliminary studies, 1930-31, the vitamin D was introduced into the ration by irradiation of the cornstarch with a Cooper-Hewitt Mercury Vapor Quartz Lamp at a distance of 28 inches for a fifteen-minute period. During the 1931-32 experiments, vitamin D was supplied to the animals as 250 D viosterol in the amount of 3 drops weekly per rat. This latter source of vitamin D proved more satisfactory as a few animals gave indications of rib beading when vitamin D was supplied through irradiation of the cornstarch.

The three varieties of apples that were tested for vitamin A were Golden Delicious and Starking (Red Delicious), both of which are yellow-tissued varieties, and the McIntosh, a white-tissued variety. The apples were obtained from one of the chief fruit districts of Washington and were kept in a cool basement at a temperature of 40-45° F., this condition being comparable to good common storage. The apples averaged three and one-half months in storage at the time of feeding. They were taken from storage weekly and kept in an electric refrigerator at a maintained temperature of 40°F. for the daily feedings. They were peeled and chopped to insure uniform sampling, weighed quickly, and fed before there was appreciable evidence of brown discoloration from oxidation. These feedings were given to the rats daily six days per week throughout the experimental period.

A preliminary study was conducted in 1930-31 on four feeding levels of apple. These levels were 0.5, 0.75, 1.0, and 1.5 gm. per rat per day, the rats being fed for a 56-day period. The following year the study was repeated with all animals placed on the 1.5-gm. feeding level. The preliminary study showed that the three lower levels of apples were below the amount required to produce the unit weight gain for vitamin A. A 35-day

experimental period was employed in 1931-32. According to the U. S. Pharmacopoeia method (9), the unit for vitamin A is that amount of food which when fed to a test rat will induce a gain in weight of 10 to 20 gm. (an average of 3 gm. weekly) in a 35-day experimental period.

All animals were weighed weekly. The negative control groups received only the basal ration, while the positive control groups were given the basal ration into which was incorporated 10 per cent butter fat. Post-mortem examinations were made at the close of the experimental periods following the methods of Sherman and Munsell (8).

#### EXPERIMENTAL DATA

The 45 rats used in the 1931-32 study averaged 44 gm. in weight when placed on the vitamin A-free basal ration for depletion of vitamin A reserves. The depletion period was approximately five weeks in length and the animals averaged 113 gm. when they were placed in individual cages at the time the apple feedings were initiated. The data of Table I show the

TABLE I

RAT GROWTH RECORDS COMPARING APPLES WITH YELLOW AND WITH WHITE TISSUE AS A SOURCE OF VITAMIN A. (1931-32)  
(35-day experimental period)

Supplement to basal diet	Amount fed daily, six days per week	Number of rats	Average weights		Average time of survival	Average loss or gain per week
			Initial	Final		
	gm.		gm.	gm.	days	gm.
Starking (Red Delicious) (yellow tissue)	1.5	9	113	125	35	+2.4
Golden Delicious (yellow tissue)	1.5	8	88	110	35	+4.4
McIntosh (white tissue)	1.5	8	107	132	35	+5.0
Positive control 10% butter fat	—	10	116	181	35	+13.0
Negative control (no test food)	—	10	112	86	19	-9.5

weight responses of the rats fed 1.5 gm. of Starking (Red Delicious), Golden Delicious, and McIntosh apples, together with those of the positive

and negative control groups. In the groups receiving apple feedings, the average weekly weight-gain was highest for the McIntosh, with Golden Delicious second, and the Starking (Red Delicious) third, though these differences were only slight. The negative control group showed marked evidence of infection, while the positive group was apparently protected in every respect.

### CONCLUSIONS

The results of this study show there is practically no difference in the vitamin A content of the varieties of apples tested. Since the McIntosh, an apple with white tissue, shows a vitamin A potency at least equal to two yellow-meated varieties (Red Delicious and Golden Delicious), it would appear that the presence of yellow pigment in the apple is not related to vitamin A potency. The unit for vitamin A, as determined for these varieties is near the 1.5 gm. level. Munsell (10) has summarized her studies on the vitamin A value of certain foods and has reported the vitamin A unit for orange juice (Florida) to be 1.5 gm. and for apples (raw, cold storage) to be 2 gm. The three varieties of apples tested in the present study were richer in vitamin A than Munsell has indicated and it is clearly shown that they are equal to orange juice in vitamin A potency and compare favorably with other fruits as a source of vitamin A.

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## EDITORIAL REVIEW

### THE CHEMICAL NATURE OF ENZYMES

**T**WO of the most important problems in biological chemistry are the chemical nature of enzymes and their mode of action. These questions are as difficult as they are important. The purification of enzymes is an extremely troublesome task, owing to their great instability, their low concentration in plant and animal tissues, and their colloidal nature. Then, too the enzyme is markedly affected by changes in temperature, differences in pH, and by various impurities which may be present. Much of the earlier work with enzymes is now known to be of little value because before the time of Sørensen (1) the significance and effects of pH were not understood.

In their work with urease Sumner and Hand (2) found it unsafe to employ ordinary distilled water, for this usually contains traces of heavy metals and these poison the enzyme. Many of the freakish actions which have been observed with urease have been due to the use of impure distilled water. For example, the enzyme has been observed to wax and wane in activity, or it has been found to require the presence of some co-enzyme (3, 4), or activator (5-11) before exhibiting its full activity. Such substances as proteins, amino acids, or hydrocyanic acid have been described in the literature as functioning as auxo-substances, activators, or promoters, and all the while the observed effects have been due simply to a poisoning of the enzyme by heavy metals which was eliminated by the substance added (2, 12).

To just what extent this is the true explanation of the action of activators on other enzymes it is still too early to say. We have the interesting example of the vegetable protease papain, which digests proteins best after treatment with hydrocyanic acid, hydrogen sulfide, cysteine, or glutathione. According to Willstätter and Grassman (13) these substances function as activators; according to Krebs (14) they act by removing ions of heavy metals.

Enzymes have been known for about one-hundred years and until the extensive researches of the Willstätter school, which began about 1918, the enzymes had been regarded by most persons as proteins. There is con-

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siderable justification for this viewpoint since enzymes have many properties similar to those of proteins. A comparison is given below.

ENZYME	PROTEIN
colloidal nature	colloidal nature
soluble in water	soluble in water
inactivated at 60–80°C.	denatured at 60–80°C.
inactivated by acid and alkali	denatured by acid and alkali
inactivated by long contact with distilled water	denatured by long contact with distilled water
inactivation reversible in some instances	denaturation reversible in some instances
reversibly inactivated by heavy metals	reversibly precipitated by heavy metals
molecular weights above 20,000	molecular weights above 20,000

However, as the result of their very extensive researches, Willstätter and his students have taken the stand that enzymes are not of protein nature and that their chemical composition is entirely unknown. Willstätter (15, 16) has claimed that the enzyme exists adsorbed to some un-specific colloidal carrier.

The Willstätter school has purified enzymes for the most part by adsorption, making use of such adsorbents as kaolin and various preparations of aluminum hydroxide (17). As stated by Willstätter and Waldschmidt-Leitz (18): "There exists but a single method for the isolation of enzymes which is versatile, adaptable, and capable of development, namely the employment of adsorption processes based upon small affinity relations, or upon residual affinities." In many instances a considerable degree of purification has been achieved by use of adsorption methods, but in no case has any enzyme been obtained in condition of purity sufficient for any conclusion to be drawn regarding its chemical composition. Thus, yeast saccharase has been concentrated some 3000 to 4000 fold by adsorption and precipitation (16); animal lipase has been concentrated about 250 fold (19); liver esterase 128 fold (20); and castor oil lipase 100 fold (21). The greatest degree of concentration was that accomplished with the enzyme peroxydase, which Willstätter and his collaborators (22, 23) concentrated 20,000 fold. Recently Kuhn, Hand and Florkin (24) repeated this work and claim to have shown that, contrary to the conclusion of Willstätter, peroxydase contains iron as an essential constituent.

Willstätter (25) states that he obtained saccharase free, or almost free, from carbohydrates, phosphorus compounds, and proteins. "*Das Ergebnis war, dass sich die Saccharase von chemisch definierbaren hochmolekularen Stoffen, wie Kohlenhydraten, Phosphorverbindungen und Proteinsubstanzen, ohne Einbuße an Aktivität, sogar an Beständigkeit, gänzlich oder fast ganz*

*befreien liess.*" However, the highly purified saccharase contained from 4 to 10 per cent of nitrogen. One wonders if this could have been protein nitrogen. Willstätter (26) found some preparations of saccharase to be negative to the Millon and the ninhydrin tests. It is true, nevertheless, that the Millon reagent is not a delicate reagent for proteins which are poor in tyrosin. Besides, in testing for an unknown chemical substance one is always confronted with the possibility that the substance may be present in concentration too small to respond.

As a result of domination by Willstätter many biological chemists have come to regard enzymes as insusceptible of isolation. However, in 1926 Sumner (27) isolated from the jack bean a crystallizable globulin which he stated to be identical with the enzyme urease. Four years later Northrop (28) starting from high-grade commercial pepsin preparations obtained crystals which he believes to be identical with pepsin. Somewhat later Northrop and Kunitz (29) were able to obtain a crystalline protein possessing a high tryptic activity, whilst Caldwell, Booher and Sherman (30) reported that they had obtained from pancreatin a crystallizable protein which exerted high amylase activity. It is yet too early to discuss the incomplete evidence regarding crystalline trypsin and crystalline amylase, but for the isolation of urease (27, 31-37) and pepsin (28, 38, 39) in the form of crystallized globulins the experimental work is now both complete and convincing.

Urease crystals are obtained in an extremely simple manner (27). One mixes jack bean meal with 32 per cent acetone and filters in an ice-chest. The next day the filtrate is found to contain the crystals, which can be centrifuged off. If one employs jack bean meal rich in urease the amount of concentration is about 730 fold. Using meal of poor quality the purification possible is 1400 fold. Naturally the number of times that a substance can be purified is limited by the original concentration and the very fact that urease crystallizes directly from extracts of jack bean meal indicates a high concentration in the meal. The concentration of urease in the jack bean ranges from 0.07 to 0.15 per cent. If the urease in the soy bean is the same substance then the soy bean contains 0.01 per cent. When purified by recrystallizing from 32 per cent acetone, or from 30 per cent alcohol, the urease activity of the crystals eventually increased to a value of about 133,000 units per gram, and this figure has not been exceeded. It is of interest to note that Waldschmidt-Leitz (40) tried to purify urease by adsorption, using jack bean meal rich in urease, and that the most active material obtained had only 25,000 units per gram.



Many erroneous statements regarding crystalline urease have appeared in scientific journals and monographs. For example, the crystals have been described as "sphero-crystals" and have been said to be prepared by "precipitation with acetone." Haldane and Stern (41), in their recent text book, figure that crystalline urease of 130,000 units per gram would decompose one-fifth of its weight of urea per second. Since a urease unit has been defined as that amount of enzyme which will produce 1 mg. of ammonia nitrogen in 5 minutes at 20°C. one can reckon that 1 gm. of urease would split 928 mg. of urea per second, or practically its own weight. Under optimum conditions crystalline urease is somewhat more active than this. Again, they state that urease crystals obtained from jack bean meal poor in urease possess about one-half of the activity of crystals obtained from the best meal, but fail to mention the fact that the impure crystals can be raised in activity by recrystallization. They doubt whether crystalline urease should be classified as a globulin since it is soluble in distilled water. However, urease is not soluble in water at its isoelectric point unless salt is present. This property, together with its ability to coagulate when heated, places it among the globulins.

Certain enzyme chemists have attempted to draw conclusions regarding the purity of different enzymes by making comparisons of the velocities of substrate decomposition caused at a given temperature by a unit weight of enzyme. On this basis Northrop's crystalline pepsin is less active than crystalline urease and the urease is far less active than the admittedly impure peroxydase of Kuhn, Hand, and Florkin (24). However, there is no reason to suppose it possible to compare two enzymes catalyzing totally different chemical reactions; nor is there any *a priori* reason to expect all enzymes to be equally efficient as catalysts.

The method for preparing crystalline pepsin (28) is a little more complicated than that for preparing crystalline urease. Parke Davis 1-10,000 pepsin is dissolved in water, acidified with sulfuric acid, and salted out with magnesium sulfate. The precipitate is dissolved by the cautious addition of alkali and then reprecipitated by acid. This precipitate is dissolved by partly neutralizing and warming. Upon cooling from 45°C. pepsin crystals form. These crystals are about six times more active than the original material. The crystals are globulin in nature and have a constant activity that is not changed materially during seven recrystallizations. When a solution of crystals is heated, or treated with alkali, the inactivation of the pepsin proceeds at the same rate as the denaturation of the protein. Recently Northrop (38) has inactivated the pepsin completely

by addition of alkali and then has partly reactivated the enzyme by bringing the reaction to a faint acidity, as was done years ago with impure pepsin by Pawlow and Parastschuk (42). Next, Northrop fractionated and concentrated the active portion and obtained pepsin crystals possessing the same activity and crystalline habit as the original crystals.

Holter (43) has adsorbed crystalline pepsin upon aluminum hydroxide and claims to have fractionated it into two portions possessing different gelatin liquefying power. In view of the discovery by Northrop (44) that pepsin preparations contain a special gelatin-liquefying enzyme, Holter's observation does not appear to indicate that the crystalline pepsin is necessarily highly contaminated with foreign material. Northrop says: "This gelatinase is present in very small quantities in the original material and is completely removed from the crystalline pepsin only with considerable difficulty."

In 1885 Sundberg (45) obtained highly active pepsin solutions which gave no test for protein. His procedure was to extract the gastric mucosa of calf stomach with saturated sodium chloride for two or three days. The filtrate was dialysed against acidified water and then allowed to autolyse for one or two weeks. The pepsin was adsorbed upon calcium phosphate and the precipitate then dissolved in 5 per cent hydrochloric acid and dialysed. The pepsin thus obtained was negative to a large number of protein reagents. However, Sundberg states that the addition of absolute alcohol caused a white precipitate to form. This precipitate contained nitrogen and when heated upon platinum foil gave a rather strong odor of burnt horn. "*Beim Erhitzen auf einem Platinbleche wurde nämlich ein ziemlich starker Geruch nach gebrannten Horn wahrgenommen.*" What better test for protein or protein degradation products could one ask?

Willstätter and Rohdewald (46) have repeated Sundberg's work and have obtained pepsin solutions which even before adsorption upon calcium phosphate gave no Millon, xanthoproteic, or ferrocyanic acid tests. They have compared the activity of this pepsin with that of Northrop's crystalline pepsin, but, unfortunately, have employed pepsin crystals which had been dehydrated with acetone and which must have become partly inactivated. Considered as evidence against the protein nature of Northrop's crystalline pepsin their results are unconvincing.

Waldschmidt-Leitz (47) has taken up the attack against crystalline enzymes and has published a paper in which he states that he has obtained pancreatic amylase entirely free from protein. In collaboration with Steigerwaldt (40) he has published an article in which he shows that crys-

talline urease is not inactivated by trypsin, as would be expected if urease were a protein, and that after 48 hours incubation with trypsin at pH 7.0 and 30°C. the protein material comprising the crystals has become so far digested that a hardly perceptible precipitate is given upon adding sulfosalicylic acid. Since the urease is not inactivated by trypsin, whilst the protein is digested, he argues that the identity of urease with the urease crystals can be no longer maintained. The results of this paper were soon accepted by Willstätter (48).

At first sight the evidence looks convincing, but it should be noticed that Waldschmidt-Leitz and Steigerwaldt employed urease in a concentration of 1 part in 67,000, and that at this concentration urease gives hardly any visible precipitate with sulfosalicylic acid even before adding trypsin. On the other hand, as noted by Sumner and Kirk (36), trypsin itself gives such a heavy precipitate with sulfosalicylic acid that one is entirely prevented from observing the precipitate due to urease. Sumner and Kirk were able to detect no hydrolysis of crystalline urease by trypsin when they weighed the protein precipitable with sulfosalicylic acid. Furthermore, by making use of the precipitin test with immune rabbit serum (antiurease) they have shown that crystalline urease diluted 67,000 times is not appreciably affected by trypsin.

In a second paper Waldschmidt-Leitz and Steigerwaldt (49) have asserted that the precipitin reaction with antiurease is not a test for protein, but that it is a test for the enzyme itself. They have carried out new digestions, using purified trypsin and allowing it to act upon crystalline urease for longer periods of time. They find that the urease activity decreases less rapidly than the amount of protein present when the latter is estimated nephelometrically. Hence, they again state that there is no connection between the urease crystals and the enzyme, except that the crystalline protein serves as an especially suitable carrier.

Sumner, Kirk, and Howell (50) deny that antiurease precipitates only the urease and not the crystalline protein. They have investigated the effect upon crystalline urease of pepsin and papain-H<sub>2</sub>S at pH 4.3 and find that while urease is slowly inactivated at this pH the presence of pepsin or papain increases the rate of inactivation many times. Under optimal conditions the inactivation of urease by active pepsin was found to be more than 20 times the rate of inactivation in the presence of boiled pepsin. Since pepsin in dilute solution gave no precipitate with dinitro-salicylic acid it was possible to follow the hydrolysis of urease turbidimetrically and to compare the rate of hydrolysis with the rate of inactivation. These two

have been found to be identical. The authors declare, therefore, that since urease is inactivated and simultaneously digested by pepsin and papain, the results of Waldschmidt-Leitz and Steigerwaldt, obtained by digesting urease with trypsin, cannot be used as an argument that urease is not a protein.

The controversy over the protein nature of urease reminds one of the similar dispute as to whether ricin, abrin, and other vegetable toxins were, or were not, proteins (51). Certain investigators (52) stated that the toxic properties were not diminished by incubation with trypsin and papain, while the digested material was found in some substances to be entirely free from protein. However, further investigation has shown ricin to be destroyed by pepsin (53) and by trypsin (54), although extraordinarily resistant to the latter enzyme, and the rate of its hydrolysis by trypsin has been shown to be identical with its rate of detoxication (55). At the present time the protein nature of the toxalbumins has found general acceptance, notwithstanding the fact that these substances, unlike urease and pepsin, have never been obtained in crystalline condition.

It would be of value and of interest to possess more data upon the effects of proteolytic enzymes on enzyme stability. However, the resistance of an enzyme to trypsin cannot be taken as final proof that the enzyme is not a protein. Nor, indeed, can the finding that urease and pepsin are proteins be taken to mean that all enzymes are proteins. Northrop (39) has attempted to digest his crystalline pepsin at pH 6.0 by both trypsin and papain. He found that the pepsin was not attacked, but instead the trypsin was rapidly inactivated, while the papain was slowly inactivated.

The question of the protein nature of urease is remotely related to the question as to the homogeneity of crystalline insulin. Dingemanse (56) adsorbed insulin on "supranorit" and then extracted with 85 per cent phenol. The amorphous product was four times more active than crystalline insulin. Because of this finding there has been some doubt as to whether crystallized insulin is identical with the hormone itself (48). Jensen (57), who believes in the identity, points out that: "Crystalline insulin has been prepared from the islet tissue of certain fishes and from pig and sheep pancreas. Insulin crystals from these various sources all possess the same activity, approximately 24 units per milligram, and all have the same sulfur content, 3.2 per cent." Moreover, the results of Dingemanse could not be repeated by du Vigneaud, Geiling, and Eddy (58), nor by Jensen and DeLawder (59). Nevertheless they are confirmed by the work of Dirscherl (60).

If enzymes are proteins they should act as antigens when injected into animals and one should be able to obtain antibodies of the type known as antienzymes from the animal's serum. The literature on enzymes contains many references to antienzyme formation, but most of the evidence submitted is far from convincing. Kirk and Sumner (37) have succeeded in obtaining antiurease by injecting crystalline urease into rabbits over long periods of time. Urease is very poisonous to mammals, for it converts their urea into ammonium carbonate and this causes death by alkalosis. As little as 0.15 mg. of crystalline urease is lethal for the average rabbit, but by using smaller doses it is possible to immunize. A highly immunized rabbit can tolerate in one day 400 lethal doses. The normal rabbit or guinea pig can be protected against urease by giving an injection of serum taken from an immune rabbit. The hen is not poisoned by injecting urease, as S. F. Howell (61) found, for the hen has only about 2 mg. of urea per 100 cc. of blood. However, injection of urease into the hen brings about the formation of antiurease just the same.

The serum of immunized animals contains antiurease. This substance inhibits the hydrolysis of urea by urease. When urease is added to immune serum a precipitate is formed. The reaction is so sensitive that urease at a final dilution of 600,000 times still gives a detectable haziness. This is the test which was alluded to when it was stated that urease continues to give the precipitin test even after long incubation with trypsin. It is of interest to note that soy bean urease is precipitated also by jack bean antiurease, from which it appears that soy bean urease is either identical with jack bean urease, or else closely related (62).

It is a very easy matter to purify antiurease (37). One adds crystalline urease to immune serum and, after keeping at 38°C. for 30 minutes, centrifuges off the precipitate of urease-antiurease. The precipitate is washed twice with dilute salt solution and is then dissolved in dilute hydrochloric acid. The acid denatures the urease, but has no effect on the antiurease. Upon neutralizing, the denatured urease precipitates, leaving the antiurease in solution. The recovery of antiurease is sometimes as high as 95 per cent.

Antiurease is probably a globulin. It is precipitated by dialysis and is inactivated by long dialysis. It is not affected by long incubation with trypsin at pH 7.0, but is rapidly inactivated by papain and papain-HCN at pH 5.0 and by pepsin at pH 4.3. Preliminary experiments indicate that antiurease unites stoichiometrically with crystalline urease.

## SUMMARY

It is the author's conviction that the proof of the identity of urease and pepsin with crystalline urease and crystalline pepsin is complete and that any hypothesis to the contrary would necessitate highly improbable assumptions. Adsorption methods have been fully tried out and have been found inadequate for the isolation of enzymes. Evidence intended to disprove the identity of urease crystals with the enzyme itself has either failed to bring proof, or has actually led to further investigations resulting in confirmation of earlier conclusions. At least two proteins have been prepared in condition of high purity, and have been demonstrated to possess intense enzymic activity. The activity has been shown to remain constant after repeated recrystallization and to be diminished by the action of agents which bring about denaturation, or digestion, at the same rate that the crystalline proteins are denatured, or digested. It would appear to the author that only a detailed investigation of the chemically active groups present in urease and pepsin can be expected to shed further light on their chemical nature.

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NUTRITIVE VALUE OF HIGH AND LOW  
CALCIUM-CARRYING WHEAT†

By

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AN EXTENSIVE study of the mineral content of Utah-grown grains has revealed a wide variation in their calcium, magnesium, potassium, iron, sulfur, and chlorine content. The quantity of these elements in wheat varies with the variety and with the soil on which the wheat was grown, and further whether it has been grown under irrigated or dry-farm conditions. The average calcium content of the Utah-grown wheats was found to be about twice that reported for the 20-year averages for the Rothamsted wheats and considerably higher than the results reported by Sherman. The discovery of these differences is responsible for the following statement made by Greaves and Hirst (9):

It is most probable that this (the high calcium content of the grains) would have a profound influence in the nutrition of farm animals which may be kept on a ration consisting primarily of grains. . . . This greater proportion of calcium to phosphorus in the Utah-grown grains should make of them a more nearly balanced food than is the case with the grains proportionately higher in phosphorus.

The present work is an attempt to evaluate, by animal-feeding tests, the importance of this difference.

Two varieties of wheat, Kota and Turkey, which vary widely in mineral content, were chosen for the work. The wheats were grown on the Nephi Dry-farm Substation on the same soil and under similar conditions. The analyses of the two wheats are given in Table I.

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TABLE I  
PERCENTAGES OF PROTEIN AND MINERALS IN KOTA AND TURKEY WHEATS

Variety of wheat	Protein	Ca	Mg	P	K	Fe	S
Kota	17.41	0.080	0.182	0.308	0.334	0.006	0.253
Turkey	14.42	0.055	0.159	0.153	0.276	0.006	0.202

Each constituent, with the exception of iron, is higher in the Kota than in the Turkey wheat.

### EXPERIMENTAL METHODS

Young albino rats, 22 to 25 days old, were placed in individual cages on experimental diets. Fresh water and food were given daily. Except for those placed in a dark room, the rats were kept in a well-lighted and ventilated room. Since wheat is low in protein, the wheat diets were supplemented with a basal diet consisting of: Casein, 24 grams; yeast, 32 grams; sodium chloride, 12 grams; and dried cabbage, 17 grams. This addition was expected to supplement the wheat, making a complete diet except for

TABLE II  
GIVING DIETS, TOGETHER WITH SUPPLEMENTS AND CONDITIONS UNDER WHICH ANIMALS WERE KEPT

Group	Diet	Additions	Light
1	Wheat, 9 Basal, 1	None	Ordinary diffused
2	Wheat, 9 Basal, 1	None	None
3	Irradiated wheat, 9 Basal, 1	None	Ordinary diffused
4	Wheat, 9 Basal, 1	2 drops cod liver oil (daily)	Ordinary diffused
5	Wheat, 9 Basal, 1	Calcium carbonate	Ordinary diffused
6	Wheat, 9 Basal, 1	2 drops cod liver oil (daily) Calcium carbonate	Ordinary diffused

vitamin D and the varying amounts of calcium and phosphorus content of the wheat.

The conditions of feeding are summarized in Table II.

In Groups 5 and 6 of both Kota and Turkey wheats, the amount of calcium carbonate added to the diet was that quantity necessary to make the calcium-phosphorus ratio of the diet 2 to 1. As the calcium and phosphorus contents of the wheats were different, the amount of calcium carbonate added was different in each case.

The animals were kept on the diet for a period of three weeks. They were then anaesthetized, the blood drawn from the heart, pooled, and analyzed for calcium according to the Clark and Collip method (4). The blood phosphorus was determined according to the Fiske and Subbarow method (7). The tibia was dissected and used for the line test as outlined by McCollum and Simmons (12). The femurs were extracted with ether and alcohol, dried and ashed (2). The percentage of bone ash was calculated on dry weight after extracting the fat, as the fat content of the bones in animals is variable. On the ash of the femurs calcium determinations were made by the Tisdall and Kramer method (17); phosphorus determinations were made according to the Fiske and Subbarow method (7).

The wheats of Group 3 (Kota and Turkey) were irradiated for 30 minutes, 1 foot distant from the Hanovia quartz mercury vapor lamp, then mixed with the basal diet and fed to the animals.

#### DISCUSSION OF RESULTS

Cowgill and co-workers (5) report results in which young rats were fed on rations where cereals furnished 62 to 93 per cent of the total calories. When the cereal content of the diet consisted of the smaller quantities the growth was exceptionally satisfactory, while in the diet containing sufficient cereal to yield 93 per cent of the calories, growth was not so satisfactory. In their experiments the cereal diets were supplemented with meat residue, salt mixture, cod liver oil, and 15 grams of fresh lettuce. In the experiment reported here the quantity of protein was not so great; neither was it of such a high biological value. Therefore, as rapid a growth as Cowgill reports could hardly be expected, although Cowgill gives no actual growth curves for making comparisons.

The growth curves for the different groups are given in Figures 1 to 6.

A close correlation exists in the growth of the animals on Turkey and Kota wheats when compared within the same groups, the only possible exception being in Group 5 where calcium carbonate supplements the two

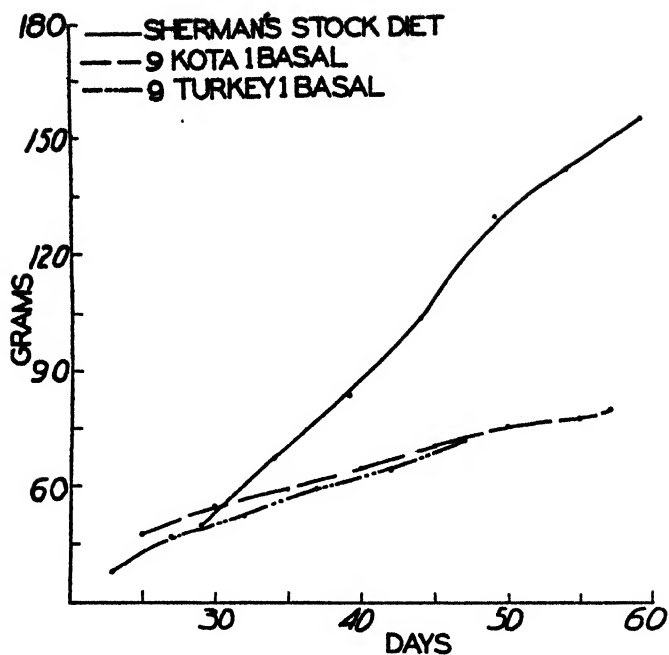


FIG. 1.

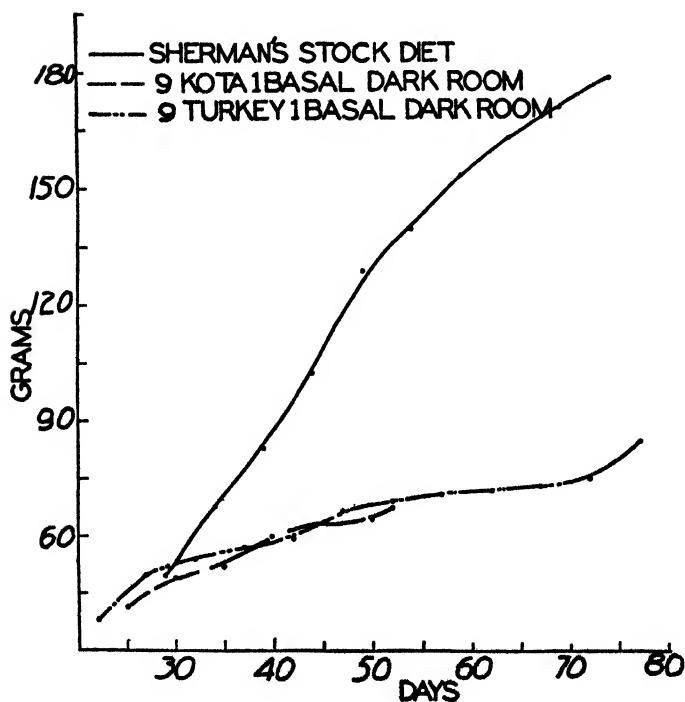


FIG. 2.

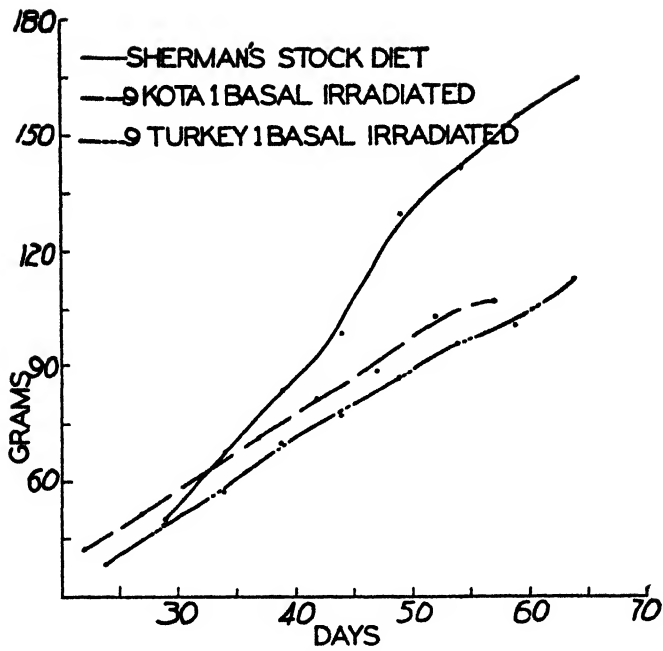


FIG. 3.

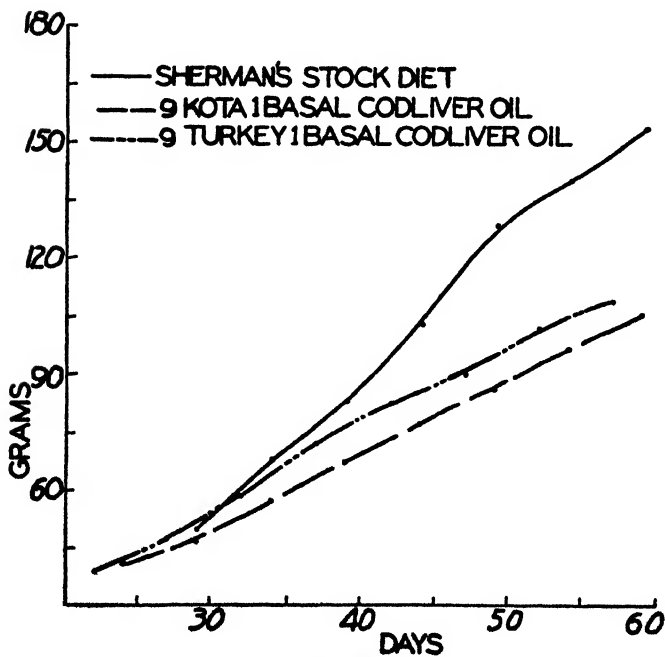


FIG. 4.

wheats. Here the better growth occurred with the Turkey. On comparing the growth in the different groups it is seen that the growth at similar ages is greater than in Group 1, indicating the presence of the mother substance of vitamin D in both wheats. This was activated to such an extent by irradiation that the growth of the animals was greatly improved. If growth is used as a measure, it may be concluded that no more activation of vitamin D occurred when the animals were kept in lighted rooms, where the sunlight had to pass through ordinary glass, than when kept in darkened rooms.

An equally good growth in these animals was obtained by irradiation of the wheat, as when cod liver oil was added to the diet. Thus, it is evident that these wheats contain a fair amount of the mother substance of vitamin D. In all groups, however, growth is not equal to that of the growth curve obtained with animals on Sherman's stock diet, but more nearly approaches this curve as the wheats are supplemented with calcium carbonate and cod liver oil. The diets of the different groups are probably deficient quantitatively in protein content as well as in the nature of amino acids making up the protein content.

The quantity of food required to make 1 gram of gain in weight is practically the same with both wheats.

TABLE III

GRAMS OF FOOD REQUIRED TO MAKE 1 GRAM GAIN IN WEIGHT (AVERAGE RESULTS OBTAINED ON FROM 7 TO 35 ANIMALS)

Grams of food	Wheat	
	Kota	Turkey
9 wheat+1 basal	6.5±0.3	6.2±0.4
9 wheat+1 basal, darkened room	7.5±1.3	7.6±0.5
9 wheat+1 basal, irradiated	4.3±0.1	4.6±0.1
9 wheat+1 basal+cod liver oil	4.5±0.2	4.9±0.1
9 wheat+1 basal+CaCO <sub>3</sub>	4.7±0.3	4.0±0.1
9 wheat+1 basal+CaCO <sub>3</sub> +cod liver oil	4.2±0.1	4.4±0.1

Animals make much better gains with both wheats after the wheats have been irradiated. In short, they make as great a gain with the irradiated wheats as they do where the grains are supplemented with cod liver oil, thus indicating that there is sufficient of the sterol in these wheats for normal growth, provided it be activated. The wheats are slightly more effective when supplemented with calcium carbonate.

Dutcher, Creighton, and Rothrock (6) established standards for differ-

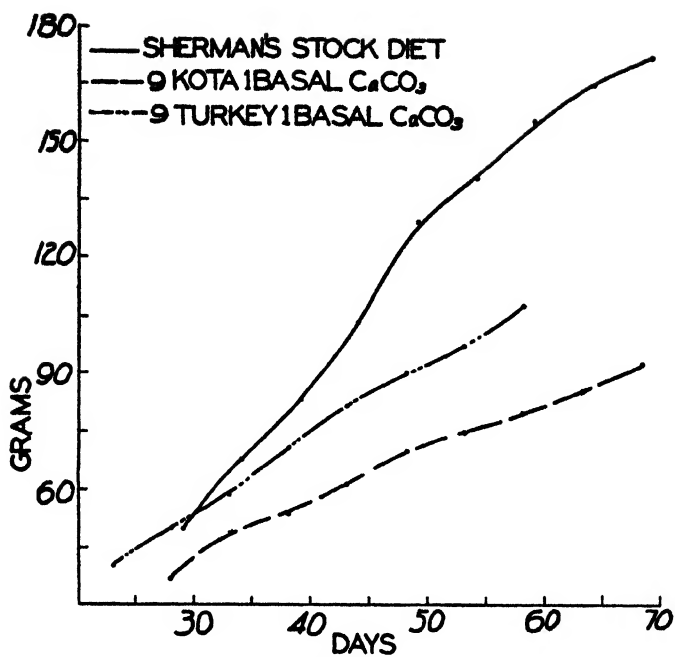


FIG. 5.

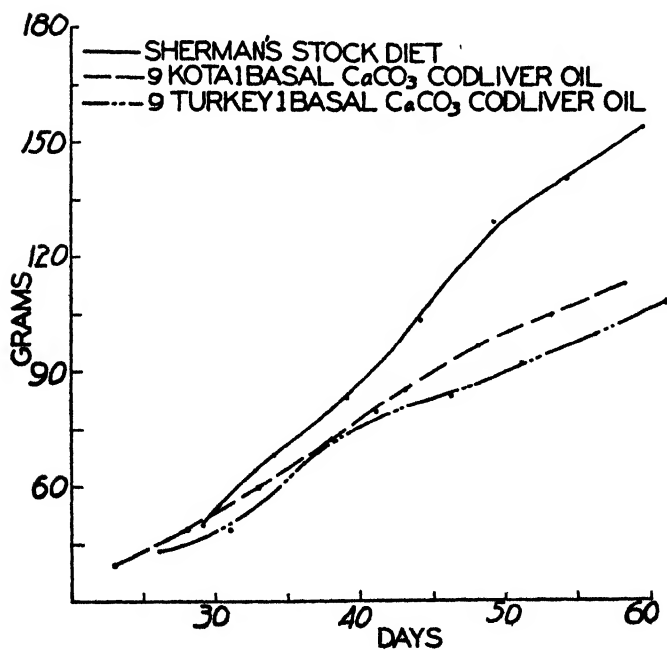


FIG. 6.

ent ages in their animals on normal and rachitic diets, using the level of the inorganic phosphorus in the blood and the percentage of bone ash calculated on the basis of dry-extracted femurs. Bethke, Steenbock, and Nelson (2) also used the percentage of bone ash, calculated on the same basis, as being indicative of the animals' conditions when fed different diets. They concluded that normal animals should have a bone ash of approximately 50 per cent. Using this as a basis of comparison, it seems apparent that the Kota wheat (as shown in Table IV) is a more nutritious wheat than the Turkey. In each of the different groups, the percentage of bone ash is higher in the Kota than in the Turkey. In Group 6 of the Kota, there is a bone ash percentage of 49.2, a normal figure; in comparison with this same group of the Turkey, there is a bone ash percentage of 32.4, a much lower figure. Both of the wheats were supplemented with cod liver oil and calcium carbonate.

In Group I, the Kota wheat yields a bone ash of 36.9 per cent. This is a low figure, as indicated above; these animals, however, are young, and younger animals have a lower bone ash. Bethke, Steenbock, and Nelson found a bone ash of 59 per cent in animals 66 days old and weighing from 200 to 275 grams. In both wheats, the bone ash of Group 2 is lower than that of Group 1, showing that some activation of sterol in animals' diet occurs in the well-lighted room. In Group 4, when 2 drops of cod liver oil were given daily to the animals, an increase in the percentage of bone ash was obtained, thus pointing to the conclusion that an increase in vitamin D over that coming from the sterol in the wheats will increase the deposition of minerals in the bone, provided these are present in the diet. Shohl (16) found the same results in feeding experiments with children. His calcium and phosphorus retention with children was 0.44 and 0.26 gram, respectively; with an increase in the calcium and phosphorus of the diet, there was a retention of 0.87 gram calcium and 0.49 gram phosphorus.

Other authors have used different means to determine whether a bone is normal. Goldblatt and Soames (8) used the percentage of calcium in the bone. They found 20.6 per cent calcium in the bones (dry weight) of animals on a diet containing a satisfactory salt mixture and furnishing ample fat-soluble vitamins and only 14.6 per cent calcium in animals receiving no fat-soluble vitamins. Those animals on Kota wheat approached more nearly the normal amount of calcium in their bones than did those animals on Turkey wheat.

In every group of Kota wheat, a higher percentage of calcium was found in the bone than was found where Turkey wheat was fed, again indicating a more nutritious wheat.

TABLE IV  
COMPOSITION OF BONES AND BLOOD OF RATS REARED ON NINE PARTS WHEAT TO ONE PART BASAL DIET UNDER VARYING CONDITIONS

Group	No. Rats	Diet	Composition	Percentage			Blood	
				Bone Ash	Ca	P	Ca	P
1	15	9 wheat; 1 basal	Kota Turkey	36.9±0.5	13.4±0.2	6.8±0.1	6.7	14.5
	10	9 wheat; 1 basal		29.4±0.6	10.0±0.2	5.0±0.1	6.5	10.0±0.1
2	10	9 wheat; 1 basal, animals in dark	Kota Turkey	34.8±0.8	12.4±0.2	6.4±0.1	6.1	6.7
	10	9 wheat; 1 basal, animals in dark		25.7±0.4	9.0±0.1	4.5±0.1	6.0	6.0
3	34	9 wheat; 1 basal, wheat irradiated	Kota Turkey	32.4±0.4	12.4±0.2	6.7±0.1	6.2±0.1	11.2±0.2
	36	9 wheat; 1 basal, wheat irradiated		30.3±0.4	10.9±0.2	5.8±0.1	6.7±0.1	11.6±0.1
4	14	9 wheat; 1 basal; 2 drops daily cod liver oil	Kota Turkey	36.4±0.8	14.6±0.3	7.6±0.2	12.2	17.0±0.8
	38	9 wheat; 1 basal; 2 drops daily cod liver oil		31.5±0.3	11.5±0.1	6.0±0.1	6.9±0.3	10.8±0.2
5	10	9 wheat; 1 basal; CaCO <sub>3</sub>	Kota Turkey	48.4±0.9	17.8±0.4	9.4±0.4	12.2	11.4
	10	9 wheat; 1 basal; CaCO <sub>3</sub>		34.6±0.3	13.3±0.1	6.6±0.1	5.6±0.2	8.3±0.1
6	10	9 wheat; 1 basal; 2 drops cod liver oil; CaCO <sub>3</sub>	Kota Turkey	49.2±0.6	17.5±0.2	8.7±0.1	10.3	17.7
	20	9 wheat; 1 basal; 2 drops cod liver oil; CaCO <sub>3</sub>		37.4±0.6	14.0±0.2	7.2±0.1	9.4±0.4	8.9±0.1



The phosphorus content of the bones was invariably higher where the animals received Kota than where they received Turkey wheat; consequently, it is evident that although these are both dry-farm wheats and were raised on the same soil, Kota wheat under all conditions builds superior bones to the Turkey wheat.

Turning to a consideration of the calcium and phosphorus content of the blood, Cavins (3) reports the normal inorganic phosphorus content of the blood serum of rats as 7 to 8.5 mg. per 100 cc. serum. In animals rendered rachitic by a diet low in phosphorus (such as McCollum's Diet No. 3143), phosphorus is found to be about 3 mg. per 100 cc. and may run as low as 2 mg.

Dutcher, Creighton, and Rothrock (6) found the normal inorganic phosphorus of the blood serum to be 10 mg. per 100 cc. for rats 3 weeks old and 8 mg. per 100 cc. in 11-week old rats. If the rats were put on a rachitic diet (No. 3965, Steenbock) at 3 weeks of age, by the time they were 6 weeks old (21 days on the experimental diet) 1.6 mg. inorganic phosphorus per 100 cc. were found in the blood serum.

The blood calcium in Groups 1 and 2 of Kota wheat is fairly low, with an extremely high inorganic phosphorus for Group 1. However, these are not impossibly high figures, as Hess, Berliner, and Weinstock (10) report the inorganic phosphorus higher than our figures. The calcium content of the wheat is high, and the phosphorus content of the wheats increases proportionately. The blood calcium in Groups 1 and 2 of the Turkey wheat is low with a lower inorganic phosphorus than in the corresponding groups of the Kota wheat. As the serum calcium increases, the serum phosphorus decreases.

The symptoms of some of the animals in Group 2 on the Turkey wheat when kept in the dark room, together with an examination of the blood, indicated tetany.

The addition of cod liver oil or calcium carbonate, either separately or together, increased the calcium content of the blood of the animals fed on Kota wheat, but it required the addition of both to the Turkey wheat diet in order to obtain a normal calcium content of the blood. The phosphorus content of the blood of all animals, with one exception, was higher where Kota was fed than where Turkey was fed.

The line test was made on the tibiae of all animals. In Group 5, on the Kota wheat, the bones showed a higher percentage of calcium and phosphorus, although they were softer than those in Groups 4 and 6. The trabeculae of the animals in Group 5 were numerous and filled with black deposits of calcium. The zone of calcification appeared to be normal. In

comparing these bones with those of Group 4 in which cod liver oil had been added to the diet, a consistent difference was apparent. The zone of calcification in the latter group was normal, calcium deposits in the trabeculae being mainly absent. The bones were extremely hard and brittle and difficult to cut.

It is evident that there is a difference in the nutritive value of the two wheats, as is evidenced by both the bone and blood composition from the wheat. Moreover, even the addition of calcium carbonate and cod liver oil to the Turkey wheat did not make it the equal of Kota wheat as mirrored by the bone and blood results. This would lead one to believe either that the native calcium of the Kota is utilized better than the added calcium carbonate, or that there is a deficiency of phosphorus in the diet which is supplied in a measure by the extra phosphorus carried by the Kota wheat.

In some countries of the Orient, where cereals furnish as much as 84 per cent of the calories of the diet, it is likely that the calcium intake of the people is low. In this country, cereals, with the exception of milk, are the first foods usually given to children; consequently, the use of cereals carrying a high calcium content, together with cod liver oil, may change these border-line cereal diets to safe and economical ones.

The desirability of an ample supply of calcium in the diet during growth is important, as has been pointed out by Sherman and Booker (15). They state that although rats may show normal growth on limited calcium intake, they may still not have the optimum amount of calcium in their body. This is fully borne out by our results. An optimum amount of calcium in the body is accompanied by earlier maturity and extends the period between maturity and senescence. It is generally recognized that during the period of early childhood there is a great demand on the part of the body for calcium, in the formation of the teeth and bony tissue.

The importance of a high phosphorus content in the diet has not been given much consideration. However, Klein and McCollum (11) find dental caries in the teeth of rats whose blood phosphorus is below 10 mg. per 100 grams of serum. Wherever the blood phosphorus is above this critical figure, the rats appear to be immune to dental caries. If this is true, Kota wheat is a still more valuable wheat due to its high phosphorus as well as to its high calcium content. The blood figure is dependent upon the level of phosphorus, calcium, and vitamins ingested in the diet. The importance of having a high vitamin content in the diet for the prevention of dental caries was also pointed out by Mellanby (18). The blood phosphorus is higher for the animals fed on Kota wheat than for those fed on Turkey,

and it was only in the case of the animals fed on Turkey wheat that the phosphorus fell below the critical figure set by Klein and McCollum (11).

In the case of adults as well as children who eat large quantities of bread, there would be an advantage in the use of flours containing a higher percentage of calcium. According to Sherman (14) and Appleton (1), the average American diet does not contain enough calcium for maintenance and especially for optimum growth. The use of high calcium bread flour, therefore, cannot be looked upon as anything other than desirable. Therefore, this problem raises three important questions: 1.—What proportion of the calcium and phosphorus of these wheats would occur in the flour? 2.—Is there a sufficient difference to render the nutritive value of the flour of one wheat greater than the other? 3.—Is it possible to breed wheats of a high calcium and phosphorus content suitable for bread flour and which can be economically produced?

#### SUMMARY

1. High- and low-calcium containing wheats were fed to rats, the wheat furnishing 90 per cent of the diet.

2. The percentage of bone ash, of calcium, and of phosphorus in the extracted bones, and the serum calcium and phosphorus were higher in all animals fed Kota (high-calcium) wheat than in those fed Turkey (low-calcium) wheat.

3. The advisability of using high-calcium wheats for both human and animal diets is indicated.

4. The question is raised as to what proportion of the calcium carried by the wheat occurs in the flour and whether it is feasible to breed high calcium-carrying wheats.

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## EFFECT OF DIET ON EGG COMPOSITION

### I. PARTIAL CHEMICAL ANALYSES OF EGGS PRODUCED BY PULLETS ON DIFFERENT DIETS

By

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THE egg of the domestic fowl being a common article of our dietary and a valuable food for convalescents and invalids, it is a matter of considerable importance to learn in what ways, and to what extent, the feed consumed by chickens may affect the composition and dietetic value of their eggs.

The diet of laying pullets and hens has an appreciable influence on the number and size of their eggs (Atwood, 1; Parkhurst, 2; Byerly, Titus, and Ellis, 3). The taste and odor of eggs and the color of their yolks may be modified by certain feeding stuffs. Onions, rape, turnips, and fresh fish, if fed in excess, impart strong flavors to the egg (Lippincott, 4). The principal natural pigment of egg yolk is xanthophyll, and by controlling the diet it is possible to markedly increase or decrease the amount of color (Palmer, 5). Capsanthin, the pigment of pimienta peppers, readily passes into the yolk (Morgan and Woodruff, 6). Certain synthetic coloring materials, such as Sudan III, when ingested by the chicken, may also pass into the egg yolk. Many fresh green feeding stuffs, such as grass, clover, and alfalfa have a tendency to cause the yolks to become more deeply colored.

Certain chemical compounds having no particularly distinctive color or odor may be found in the egg, if ingested with the usual feed. When benzoic acid is fed to chickens, it may be found later in their eggs (Carter, Howe, and Mason, 7). The percentage of iodine in the egg may be appreciably increased by feeding iodine compounds (Scharrer and Schrop, 8; Straub, 9; Jaschik and Kieselbach, 10). There is some evidence that the iron content of eggs may be increased by feeding certain feeds rich in iron (Hartung, 11; Hoffmann, 12) but on the other hand it has not been found

possible to increase it by feeding an iron supplement containing as much as 50 mg. of iron daily (Elvehjem, Kemmerer, Hart, and Halpin, 13).

The vitamin content of eggs also may be influenced by the feed consumed. By feeding chickens diets rich in vitamin A, eggs may be produced which are unusually rich in this factor (Bethke, Kennard, and Sassaman, 14). In a later paper on the effect of diet on egg composition data will be presented to show that the vitamin B content of eggs may be greatly influenced by diet. On the other hand the writers have observed little, if any, difference in the vitamin G content. By irradiating hens one may cause the vitamin D content of their eggs to be increased (Hughes, Titus, and Moore, 15; Hart and coworkers, 16).

Although it has been found that the vitamin content of eggs may be influenced by diet and that certain chemical compounds, some having distinctive colors and tastes, may be made to occur in eggs by ingesting them with the feed, there is but little evidence to show that the percentage of crude protein and ether extract may be influenced by diet. Working with pigeons, Pollard and Carr (17) and Gerber and Carr (18) have obtained evidence that diet may affect the composition of the eggs of this species. It is the purpose of this paper to present evidence that the composition of chicken eggs may be influenced by diet.

#### EXPERIMENTAL MATERIAL

Rhode Island Red pullets of known pedigree were used. They were selected from a large number of pullets the ancestors of which had been bred for high egg production and uniformity of egg size for at least 7 generations. In making the selection a number about 50 per cent greater than that finally placed on experiment was taken. After eliminating the heaviest and lightest pullets the remaining ones were distributed among the several pens on the basis of live weight. This distribution was so made that the average weight of the pullets in any one pen differed from that of the pullets in any other of the pens by less than 50 grams and the standard deviation of the live weights was essentially the same for all pens.

The pullets were housed in a series of pens in two long, concrete-floored laying houses and were allowed the freedom of concrete-floored run-yards. The diets studied were fed from September 3, 1929, to August 12, 1930. At intervals of four weeks, beginning October 2, 1929, all eggs laid during a single day by the pullets in each of the pens were saved and analyzed. At the beginning of the experiment there were 40 pullets, each, on diets 2, 5, 8, and 9, and 25 pullets, each, on diets 10, 11, and 12.

THE DIETS

Most of the diets were compounded as follows:

	Parts by weight
Basal feed mixture.....	75
	Parts by weight
Yellow corn meal.....	500
Wheat bran.....	245
Rolled oats.....	150
Alfalfa leaf meal.....	55
Mineral mixture.....	5
	Parts by weight
Ground limestone.....	50
Steamed bone meal.....	30
Anhydrous sodium sulphate.....	10
Common salt.....	10
<i>Special supplement</i> .....	20
Cod-liver oil.....	2

The special supplements in the case of four of the diets, for which data are here presented, were:

- Diet 2: Crab meal (the dried and ground offal of the crab fishing industry);
- Diet 5: "Yeast Foam" (a dried yeast preparation sold for animal feeding purposes);
- Diet 8: A mixture of 8 parts of desiccated meat meal (about 72 per cent protein), 7 parts of North Atlantic fish meal (dried and ground white fish offal), and 5 parts of dried buttermilk;
- Diet 9: No special supplement, an additional 20 parts of the basal feed mixture were used.



The other three diets were made up as follows:

Diet No.	Pearled hominy and desiccated meat meal*	Mineral mixture**	Yeast†	Autoclaved yeast	Cod-liver oil
	Parts by weight	Parts by weight	Parts by weight	Parts by weight	Parts by weight
10	95	5	0	0	2
11	80	5	15	0	2
12	80	5	0	15	2

\* In such proportions that the diet contained 20 per cent of crude protein before the addition of the cod-liver oil.

\*\* The same as that used in diets 2, 5, 8, and 9.

† The same as that used in diet 5.

Several chemical analyses were made of each diet during the course of the investigation. The average results are given in the following table.

AVERAGE RESULTS OF PARTIAL CHEMICAL ANALYSES OF THE DIETS

Diet No.	Moisture	Crude ash	Crude protein	Ether extract	Crude fiber
	Per cent	Per cent	Per cent	Per cent	Per cent
2	11.25	11.23	15.64	5.33	5.80
5	11.41	6.23	11.21	6.57	4.13
8	11.45	6.56	20.62	6.27	3.52
9	10.84	5.77	11.53	6.10	5.00
10	9.94	6.97	19.95	5.14	1.05
11	10.08	6.48	19.47	5.09	1.31
12	9.56	6.48	20.01	5.23	1.31

### *The Average Composition of the Eggs*

The yolks were separated from the whites as carefully as possible. All the eggs laid during a single day by the pullets in each pen were used in preparing the samples. Most of the samples, for which data are here given, contained not less than five eggs and in several cases the samples were derived from as many as twenty-two eggs. The essential analytical data are presented in Tables I, II, III, IV, and V.

TABLE I  
AVERAGE DRY MATTER CONTENT OF THE YOLKS OF THE EGGS PRODUCED BY THE PULLETS ON THE SEVERAL DIETS

Date eggs were laid	Diet 2		Diet 5		Diet 8		Diet 9		Diet 10		Diet 11		Diet 12	
	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent
1929														
Oct. 2	6.05	51.2	6.66	52.0	6.28	50.3	5.54	51.1	—	—	—	—	—	—
Oct. 30	7.58	52.2	7.24	52.1	7.37	51.6	7.58	50.8	—	—	7.44	49.9	—	—
Nov. 27	6.86	49.1	7.77	55.9	8.10	52.9	7.56	52.3	7.61	51.9	7.94	50.7	8.02	52.2
Dec. 25	7.52	48.4	7.48	48.6	7.63	48.9	6.94	47.4	—	—	7.54	47.5	—	—
1930														
Jan. 22	9.37	55.6	8.74	55.3	9.02	53.1	8.24	53.8	—	—	8.35	52.9	—	—
Feb. 19	—	—	—	—	9.68	55.3	—	—	—	—	—	—	9.63	54.4
Mar. 19	8.95	51.5	8.97	52.0	9.18	52.1	9.27	52.9	8.97	53.4	9.06	53.3	8.58	52.3
Apr. 16	8.75	51.1	9.07	52.5	9.22	53.6	8.98	52.5	8.13	52.0	8.96	52.3	8.42	51.5
May 14	9.08	52.4	8.38	51.8	9.28	52.7	8.13	51.6	9.46	52.7	9.13	53.0	8.98	51.8
June 11	—	—	—	—	—	—	8.25	52.3	7.95	46.0	7.17	48.3	8.41	51.5
July 9	—	—	—	—	9.33	53.4	8.76	52.5	—	—	7.95	52.9	9.40	54.3
Mean	8.02 ±0.308	51.4 ±0.0565	8.04 ±0.216	52.5 ±0.582	8.51 ±0.253	52.4 ±0.407	7.93 ±0.248	51.7 ±0.396	8.42 ±0.267	51.2 ±1.034	8.17 ±0.179	51.2 ±0.529	8.78 ±0.162	52.6 ±0.350
σ*	1.29 ±0.218	2.37 ±0.400	0.91 ±0.153	2.44 ±0.412	1.18 ±0.179	1.91 ±0.287	1.16 ±0.175	1.85 ±0.280	0.89 ±0.189	3.43 ±0.731	0.79 ±0.126	2.35 ±0.374	0.64 ±0.115	1.37 ±0.248
C of V	16.11 ±2.786	4.61 ±0.779	11.27 ±1.924	4.65 ±0.786	13.92 ±2.140	3.64 ±0.550	14.64 ±2.255	3.59 ±0.396	10.51 ±2.298	6.70 ±1.435	9.70 ±1.557	4.59 ±0.731	7.26 ±1.315	2.61 ±0.471

\*  $\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 2}}$ ; this formula was used because of the small number of cases involved.

**TABLE II**  
**AVERAGE PROTEIN CONTENT OF THE DRY MATTER OF THE YOLKS OF THE EGGS**

Date eggs were laid	Diet 2	Diet 5	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Oct. 2, 1929	33.6	31.8	33.1	30.9	—	—	—
Oct. 30, 1929	33.5	32.4	32.3	31.6	—	—	—
Nov. 27, 1929	32.7	32.2	31.2	31.7	—	—	—
Dec. 25, 1929	32.8	32.1	32.4	32.2	—	32.4	—
Jan. 22, 1930	31.6	30.8	32.0	31.7	—	32.5	—
Feb. 19, 1930	—	—	31.9	—	—	—	31.7
Mar. 19, 1930	31.8	30.8	31.7	30.6	31.2	31.0	31.2
Apr. 16, 1930	32.7	30.7	31.6	30.3	31.4	31.9	31.3
May 14, 1930	32.3	31.3	31.3	32.1	32.1	31.6	—
June 11, 1930	—	—	—	31.7	30.0	31.2	32.3
July 9, 1930	—	—	31.4	31.5	30.0	30.3	31.3
Mean	32.6 ±0.18	31.5 ±0.18	31.9 ±0.13	31.4 ±0.14	30.9 ±0.32	31.6 ±0.22	31.6 ±0.16
σ*	0.77 ±0.130	0.75 ±0.128	0.62 ±0.094	0.67 ±0.100	1.06 ±0.227	0.87 ±0.156	0.52 ±0.112
C of V	2.37 ±0.400	2.39 ±0.403	1.95 ±0.294	2.12 ±0.320	3.44 ±0.735	2.74 ±0.494	1.66 ±0.354

**TABLE III**  
**AVERAGE FAT (ETHER EXTRACT) CONTENT OF THE DRY MATTER OF THE YOLKS OF THE EGGS**

Date eggs were laid	Diet 2	Diet 5	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Oct. 2, 1929	60.6	64.3	61.2	62.0	—	—	—
Oct. 30, 1929	58.6	59.1	60.0	60.8	—	59.6	—
Nov. 27, 1929	55.2	58.1	58.5	59.6	59.4	59.9	62.3
Dec. 25, 1929	—	—	56.3	55.7	—	52.3	—
Jan. 22, 1930	—	—	57.3	56.3	—	55.0	—
Feb. 19, 1930	—	—	61.9	—	—	—	56.9
Mar. 19, 1930	—	—	59.3	60.0	58.5	55.1	60.1
Apr. 16, 1930	—	—	61.7	61.8	59.1	58.6	59.0
May 14, 1930	—	—	59.5	56.3	54.7	57.2	54.7
June 11, 1930	—	—	—	—	57.0	55.9	57.6
July 9, 1930	—	—	61.3	60.9	60.0	57.4	60.0
Mean	58.1 ±1.50	60.5 ±1.83	59.7 ±0.43	59.3 ±0.60	58.1 ±0.60	56.8 ±0.59	58.7 ±0.70
σ*	3.86 ±1.603	4.71 ±1.296	2.02 ±0.305	2.67 ±0.424	2.19 ±0.427	2.63 ±0.418	2.73 ±0.492
C of V	6.64 ±1.836	7.78 ±2.155	3.39 ±0.512	4.50 ±0.717	3.77 ±0.735	4.63 ±0.738	4.65 ±0.840

$$r = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 2}}; \text{ this formula was used because of the small number of cases involved.}$$

TABLE IV  
AVERAGE DRY MATTER CONTENT OF THE WHITES OF THE EGGS PRODUCED BY THE PULLETS ON THE SEVERAL DIETS

Date eggs were laid	Diet 2		Diet 5		Diet 8		Diet 9		Diet 10		Diet 11		Diet 12	
	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent	Grams	Per cent
1929														
Oct. 2	3.33	12.0	3.84	12.6	3.54	12.7	3.42	12.6	—	—	—	—	—	—
Oct. 30	3.49	12.6	3.38	12.9	3.98	13.3	3.74	13.1	—	—	4.09	13.5	—	—
Nov. 27	3.39	12.2	3.84	12.7	3.66	13.1	3.53	12.7	3.54	11.8	3.56	12.4	3.79	12.3
Dec. 25	—	—	—	—	3.19	10.5	2.95	10.8	—	—	3.26	10.2	—	—
1930														
Jan. 22	—	—	—	—	3.69	12.0	3.58	12.1	—	—	4.18	11.7	—	—
Feb. 19	—	—	—	—	4.24	13.1	4.13	12.5	—	—	3.86	11.9	3.34	11.7
Mar. 19	—	—	—	—	4.44	13.2	4.00	12.4	4.37	12.7	3.96	12.2	4.34	12.6
Apr. 16	—	—	—	—	3.95	12.1	4.05	11.8	3.88	11.6	3.65	10.7	3.94	12.7
May 14	—	—	—	—	3.75	11.9	3.42	11.5	3.88	13.1	3.84	11.6	4.25	12.4
June 11	—	—	—	—	4.22	12.2	3.84	12.0	—	—	3.89	12.3	3.84	10.9
July 9	—	—	—	—	4.13	12.2	4.19	12.7	—	—	3.23	10.9	3.16	10.6
Mean	3.40 ±0.045	12.2 ±0.168	3.69 ±0.146	12.7 ±0.084	3.89 ±0.078	12.4 ±0.174	3.71 ±0.081	12.2 ±0.140	3.92 ±0.141	12.3 ±0.296	3.75 ±0.073	11.7 ±0.217	3.81 ±0.121	11.9 ±0.236
$\sigma^*$	0.11 ±0.031	0.43 ±0.119	0.38 ±0.103	0.22 ±0.060	0.38 ±0.055	0.86 ±0.123	0.40 ±0.057	0.69 ±0.098	0.42 ±0.100	0.88 ±0.029	0.34 ±0.052	1.02 ±0.153	0.48 ±0.086	0.92 ±0.167
C of V	3.36 ±0.976	3.52 ±0.970	10.19 ±2.835	1.70 ±0.463	9.87 ±1.433	6.96 ±1.066	10.69 ±1.555	5.64 ±0.814	10.68 ±2.576	7.13 ±1.733	9.14 ±1.390	8.66 ±1.316	12.51 ±2.290	7.77 ±1.409

\*  $\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 2}}$ ; this formula was used because of the small number of cases involved.

TABLE V  
AVERAGE PROTEIN CONTENT OF THE DRY MATTER OF THE WHITES OF THE EGGS

Date eggs were laid	Diet 2	Diet 5	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Oct. 2, 1929	87.2	87.8	88.2	—	—	—	—
Oct. 30, 1929	88.8	88.6	87.9	88.1	—	88.9	—
Nov. 27, 1929	90.5	89.7	89.2	88.9	90.1	90.2	89.7
Dec. 25, 1929	—	—	88.3	87.4	—	87.1	—
Jan. 22, 1930	—	—	87.8	87.8	—	87.4	—
Feb. 19, 1930	—	—	88.4	88.0	—	88.7	88.7
Mar. 19, 1930	—	—	90.0	87.9	87.4	88.9	88.4
Apr. 16, 1930	—	—	88.4	87.0	87.2	87.7	88.4
May 14, 1930	—	—	87.1	88.1	89.6	89.1	88.9
June 11, 1930	—	—	88.2	87.8	87.8	88.4	88.8
July 9, 1930	—	—	87.7	88.4	—	88.7	88.2
Mean	88.8 ±0.91	88.7 ±0.53	88.3 ±0.16	87.9 ±0.12	88.4 ±0.47	88.5 ±0.21	88.7 ±0.14
σ*	2.34 ±0.644	1.35 ±0.372	0.81 ±0.117	0.55 ±0.083	1.54 ±0.329	0.97 ±0.146	0.54 ±0.098
C of V	2.63 ±0.725	1.53 ±0.421	0.92 ±0.132	0.62 ±0.094	1.74 ±0.371	1.09 ±0.164	0.61 ±0.110

\*  $\sigma = \sqrt{\frac{\sum(x-\bar{x})^2}{n-2}}$ ; this formula was used because of the small number of cases involved.

## DISCUSSION

Inasmuch as the pullets to be used in this study were to be fed in groups, the experimental work was planned and carried out in such a way as to avoid, as far as possible, the effect of individuality. To this end special attention was given 1.—to the homogeneity of the "population" from which the experimental birds were selected, 2.—to the manner of distributing the pullets among the several groups, and 3.—to the size of the groups and the method of obtaining the egg samples from the several groups.

The pullets were taken from an unusually homogeneous "population." As previously stated, their ancestors had been bred for high egg production and uniformity of egg size for at least seven generations. To insure homogeneity in live weight, as well as in breeding, all heavy and all light in-

dividuals were discarded before the pullets were distributed among the several pens.

The distribution of the pullets among the pens was so made that, at the start, the average live weight of the pullets in any one pen was within 50 grams of the average live weight of those in any other pen and the standard deviation of the live weights was essentially the same for all pens.

A sufficiently large number was placed in each pen to insure obtaining enough eggs for a suitable analytical sample on any one day after the pullets began to lay. The collection of eggs for these samples was made at regular intervals of four weeks and the number of eggs included in most of the samples varied from five to twenty-two. That the samples thus taken were random, in so far as the group from which they were obtained is concerned, is indicated by the fact that no single pullet contributed to more than 7 of the 11 samples from any one pen and, on the average, less than one-fourth of the pullets contributed to more than 3 samples.

Thus it is evident that the several groups were as similar as it was possible to make them and that any individuality which may have been originally inherent in any group could not have exhibited itself uniformly, or even approximately so, in all eleven samples. It now remains to ascertain whether, or not, the differences found were due to chance alone or to the diets.

The usual method of comparing means and their probable errors shows that there were no statistically significant differences between the eggs laid by the pullets receiving the several diets, in the case of:

- (a) the weight of dry matter in the yolks,
- (b) the percentage of dry matter in the yolks,
- (c) the percentage of fat in the dry matter of the yolks,
- (d) the weight of dry matter in the whites,
- (e) the percentage of dry matter in the whites, and
- (f) the percentage of protein in the dry matter of the whites.

However, this method does show that the eggs laid by the pullets on diet 2 (the diet which contained 20 parts of crab meal) had a significantly higher percentage of protein in the dry matter of their yolks than did the eggs from the pullets on diets 5, 9, 10, 11, and 12, the differences being from 3.6 to 5.2 times their probable errors. Had there been a sufficiently large number of samples on which fat determinations had been made, it is very probable that here, too, differences could be demonstrated.

Although the usual method of comparing means and their probable errors is applicable, when it is desired to show differences in average composition, it is not wholly suitable in the present case because of the rela-

tively small number of analyses. For the present purpose "Student's" method is the more appropriate one to use. This method is applicable when a number of paired values are to be compared. In this investigation the

TABLE VI  
TABULATION OF THE COMPARISONS MADE BY "STUDENT'S" METHOD

Comparison	The chance of obtaining a difference equal to, or greater than, the one observed, if, a priori, no difference in the composition of the eggs is to be expected	Comparison	The chance of obtaining a difference equal to, or greater than, the one observed, if, a priori, no difference in the composition of the eggs is to be expected
Weight of dry matter in the yolks		Percentage of dry matter in the yolks	
8 > 2	1 in 10	5 > 9	1 in 28
8 > 5	1 in 16	10 > 2	1 in 29
8 > 9	1 in 213**		
8 > 10	1 in 5		
8 > 11	1 in 25		
8 > 12	1 in 21		
Percentage of protein in the dry matter of the yolks		Percentage of fat in the dry matter of the yolks	
2 > 5	1 in 3500***	5 > 2	1 in 35
2 > 8	1 in 100**	8 > 2	1 in 27
2 > 9	1 in 178**	9 > 2	1 in 55
8 > 12	1 in 38	8 > 11	1 in 184**
11 > 5	1 in 29	9 > 10	1 in 81*
		9 > 11	1 in 79*
Weight of dry matter in the whites		Percentage of dry matter in the whites	
5 > 2	1 in 7	5 > 2	1 in 76*
8 > 2	1 in 32	8 > 2	1 in 233**
9 > 2	1 in 26	9 > 2	1 in 370**
8 > 9	1 in 308**	8 > 11	1 in 240**
Percentage of protein in the dry matter in the whites			
11 > 9	1 in 199**		

\* These values are on the borderline of significance.

\*\* These values are significant.

\*\*\* This value is distinctly significant.

composite samples of eggs collected during a single day once every four weeks yielded a number of series of paired analytical data.

All possible comparisons were made and those which revealed statistically significant differences, and a few which did not, are tabulated in Table VI. The rows of the tabulation are read as follows (as examples, (a) the third row under "weight of dry matter in the yolks," and (b) the first row under "percentage of protein in the dry matter in the yolks"): (a) "The eggs resulting from diet 8 had more dry matter in their yolks than those from diet 9. There is only one chance in 213 that this or a greater difference could have occurred due to chance alone." (b) "The eggs resulting from diet 2 had a greater percentage of protein in the dry matter of the yolks than those from diet 5. There is only one chance in approximately 3500 that this or a greater difference could have occurred due to chance alone."

*A priori*, one might expect to find that eggs produced by pullets receiving a well balanced diet (diet 8) would be different in composition from those resulting from a diet derived almost exclusively from plant sources (diet 9), or from a diet of very simple composition (diet 11). Such appears to be the case, for the eggs resulting from diet 8 contained more dry matter in the yolks than those from diet 9. They also contained a higher percentage of fat in the dry matter of the yolks than those from diet 11; and more dry matter in the whites than those from diet 9. A study of Table VI shows that the eggs laid by the pullets receiving diet 8 differed in several ways from the eggs resulting from the other diets.

The effect of diet 2 on the composition of the eggs is noteworthy, in view of the fact that this diet contained only a single source of animal protein, i.e., crab meal. The eggs resulting from feeding this diet had a greater percentage of protein but a smaller percentage of fat in the dry matter of the yolks than those from diets 5, 8, and 9; they also had a smaller percentage of dry matter in the whites.

The eggs resulting from the feeding of the diet derived chiefly from plant sources (diet 9) contained more fat in the dry matter of the yolks than those from diets of much simpler composition (diets 10 and 11), but they contained less protein in the dry matter of the whites.

In general, diet seemed to have less effect on the composition of the whites than it did on that of the yolks.

It is obvious from the comparisons tabulated in Table VI that differences in the composition of eggs were brought about by feeding different diets to the pullets producing the eggs. That such differences may be caused by diet is noteworthy. In the second paper of this series a sensitive



biological method will be described by means of which it is possible to demonstrate other differences.

### SUMMARY

Composite samples of eggs laid on the same days by pullets of the same age and breeding but receiving different diets were analyzed, and by means of a suitable statistical method it was shown that the composition of these eggs was affected by diet.

The eggs produced by pullets receiving a well balanced diet differed in composition from eggs resulting from the feeding of less satisfactory diets.

The yolks appeared to be more readily affected by diet than the whites.

The most consistent difference, although it was small, was observed between the percentage of protein in the dry matter of the yolks of eggs laid by pullets receiving a diet containing crab meal and those receiving the other diets. This difference was demonstrable by the usual method of comparing means and their probable errors, as well as by "Student's" method.

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# THE EFFECT OF DAIRY MANUFACTURING PROCESSES UPON THE NUTRITIVE VALUE OF MILK

## II. THE APPARENT DIGESTIBILITY OF FRESH WHOLE MILK AND OF POWDERED WHOLE MILK

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GROWTH experiments in this laboratory in which albino rats were fed fresh whole milk, evaporated milk, and powdered whole milk raised a question concerning the effect of manufacturing processes upon the digestibility of milk. Among the factors involved in these processes which may possibly affect digestibility are heating, homogenization, concentration by evaporation of water, and drying. Heating is involved in most of the manufacturing processes.

Many studies of the effect of heat upon the chemical compounds of milk have been made. Before the development of modern methods of pasteurization, comparisons of cooked and sterilized milk with raw milk were a popular subject of study. Later many investigations of the effect of pasteurization were conducted. These experiments consisted largely of studies of changes in chemical composition and changes effected in digestibility as determined by means of artificial digestive fluids and by employment of animals with stomach fistulas or of human subjects from whom the stomach contents were removed by means of stomach pumps. Experiments of this sort undoubtedly yield valuable information, but they do not necessarily present a complete or reliable picture of the normal digestive process in its entirety. In the normal process of digestion, the food is first subjected to enzymic action in an acid medium. Later the resulting digestive products are acted on by a different set of enzymes in a neutral medium. During these processes the end-products are removed. It is difficult to reproduce these conditions in experiments conducted *in vitro*. Food residues which are recovered from the stomach give but a partial picture of the complete, normal process. There is no certainty that experiments conducted *in vitro* will yield results comparable with the complete life process. The variation between the results of the extensive experiments of Wallen-Lawrence and

Koch (23) with evaporated and raw milk carried out in vitro and the results recently reported by the authors (Nevens and Shaw, 14) of experiments with animals is an illustration of this lack of agreement.

So far as the authors have been able to discover only a few determinations of the completeness of digestibility of the constituents of milk have been made in experiments involving the normal, entire digestive process.

The earliest studies of the digestibility of cows' milk which have come to the authors' attention are those of Camerer (3), Prausnitz (15), Rubner (17), and Forster (5). Each of the first three of these investigators reports a three-day or four-day experiment upon a human subject receiving milk as the only food. Presumably the milk was raw except in Prausnitz' experiment, in which it was sterilized by heating for two hours in a steam sterilizer. The coefficients of apparent digestibility calculated from the data given by the three investigators, respectively, are as follows: total nitrogen, 94.3, 91.7, and 93.5; fat, 97.2, 94.9, and 95.1; dry matter, 92.9, 90.8, and 92.1. It will be observed that the coefficients for the sterilized milk are slightly lower than those for the raw milk. Both Rubner and Prausnitz state that protein and sugar could not be found in the feces. Forster found that a child failed to digest only 6.35 per cent of the dry substance of milk. The dry feces contained 34 per cent ash and 30 to 40 per cent fat.

Rubner (18) later conducted another experiment with a man weighing 70 kgm. during a two-day period in which the man consumed 2,500 cc. of milk daily, and also a one-day period in which he consumed 3,000 cc. He reports the undigested constituents of the milk for the two periods to be, in percentage, respectively: nitrogen, 7.02 and 12.91; fat, 2.57 and 7.12; dry matter, 5.72 and 11.16; sugar, 0 and 0.

Wasileff-Petersburg (25) conducted digestion experiments with six healthy young people 18 to 23 years of age. He found that the proteins of raw milk were more completely digested than those of cooked milk. A larger quantity of volatile fatty acids was found in the excreta after drinking cooked milk than after taking raw milk, leading to the conclusion that cooking lowers the digestibility of the fat.

Raudnitz (16) using a dog during three-day and four-day experimental periods found cooked milk slightly less digestible than raw milk.

Terroine and Spindler (22) employed pigs weighing 8 to 10 kgm. in their investigations of three methods of pasteurization. Their experiments showed no differences in the digestibility of the proteins or mineral constituents of the pasteurized and unpasteurized milk.

Several experiments have been carried out showing the effect of heat on

the efficiency of utilization of the components of milk. Washburn and Jones (24) and Magee and Harvey (10) found that the proteins of heated milk were less efficiently utilized by pigs than the proteins of raw milk.

Morgan (12) obtained similar results with rats in a comparison of raw casein and casein toasted at 150° to 200°C. for 30 to 45 minutes. The biological value was lowered although the digestibility was changed only a little.

Miyawaki, Kanazawa, and Kanda (11), using white rats as experimental animals, found coefficients of apparent digestibility of the proteins of four brands of dried milk ranging from 86 to 91. When the coefficients were obtained in artificial digestion experiments with pepsin, they ranged from 78 to 92, and 10 coefficients for raw milk had an average of 84.4.

The results of numerous experiments conducted *in vitro* lack uniformity. This lack of agreement may be explained in part by lack of uniformity in the intensity and duration of the heating process, in the condition of the milk before heating, and in the methods employed in the digestion processes. A few of the experiments are given brief mention.

Fleischman and Morgen (4) found that the longer milk was heated above 85°C. the less digestible the proteins became.

Baginsky (2) reports that in the case of completely sterilized milk the casein was less readily precipitated by rennin and the digestibility in gastric juice was lower than in the case of raw milk.

Somerville (19) in a series of 30 pancreatic digestion experiments found that the protein of milk reconstituted from powder was more completely digested than raw milk. These results coincided with his earlier results using pepsin.

Krull (9) reported the digestibility of the components of milk dried by the roller process to be: protein, 95.5 per cent; lactose, 99 per cent; fat, 97 per cent.

Stassano and Talarico (20) found that dry milk was more completely digested during tryptic digestion than raw milk.

Aviragnet, Dorlencourt, and Bloch-Michel (1) showed by experiments *in vitro* that milk dried by heating rapidly, at a high temperature, is more completely digested by trypsin than is raw milk.

Grimmer, Kurtenacher, and Berg (7) found that heating milk at 60°C. rendered the lactalbumen less soluble and less digestible.

Hess, Koch, and Sennewald (8) state that "boiling milk definitely reduces its soluble protein content but facilitates peptic digestion."

Gratz (6) reports no changes in digestibility of milk brought about by pasteurizing for 30 minutes.

## EXPERIMENTAL METHODS

The experiments reported in this paper deal only with studies of the completeness of digestibility of cows' milk. It is assumed that the differences between the amounts of protein, fat, sugar, and total solids ingested and the amounts of these appearing in the feces represent the amounts digested. In making this assumption, it is recognized that products of endogenous origin are present in the feces. Since this is an entirely normal and necessary part of the digestion process, it is reasonable to assume that the loss of these substances in the feces is one of the costs of digestion. Further, the extent of the loss is undoubtedly closely related to the amount and character of the particular food or mixture of foods ingested. It is believed, therefore, that the assumption that the digested nutrients are equivalent to ingested nutrients minus feces nutrients, which is the method of calculation commonly used in both human and animal nutrition, gives a highly reliable picture of the completeness of digestibility. The coefficients obtained by this method are often termed coefficients of "apparent digestibility."

Albino rats were used in these feeding tests. They were paired according to sex and approximate weights. The methods of feeding and management which were employed in previous experiments (Nevens and Shaw, 13, 14) were followed as far as possible. The rats were confined individually in glass cages below which pans lined with heavy filter paper were placed to receive the excreta. The fresh milk was fed in glass sponge dishes. For the powdered milk a modified McCollum feeding device was used. Practically none of the powder was spilled from these devices; in the few cases in which spilling did occur the milk was carefully collected and returned. The animals fed powdered milk were supplied with distilled water. Since it was impracticable to attempt to equate food intakes in this experiment, food was supplied *ad libitum*.

Fresh milk was fed twice daily. It was secured from the University Dairy Barns daily and each lot was sampled before feeding. Analyses were made of a composite sample. The milk used in Periods 1, 2, 4, and 5 consisted each day of the mid-portion of a single milking of an individual cow. The term "whole milk" as used in this and previous papers (Nevens and Shaw, 13, 14) refers to milk obtained in this manner. The milk supply for Periods 3 and 6 was secured from the mixed milk of four cows. The term "fresh" refers to unheated milk in excellent condition and not over 24 to 28 hours old when fed.

Two commercial brands of powdered milk were used, one being manufactured by the "spray" process and the other by the "roller" process.

A sufficient amount of powdered milk to supply each animal during the entire digestion trial was weighed into numbered glass jars. A sample for analysis was taken during the weighing. The powdered milk was placed in the feed dishes daily or often enough to keep a supply before the animals.

The digestion periods were each ten days in length. These trials were preceded by a preliminary period of three to five days, during which the particular experimental food to be tested was fed. Before each new digestion trial, stock mixture was fed for several days in order to keep the animals in good physical condition.

Feces were removed from the collection pans daily. In the protein digestion trials they were transferred directly to stoppered Kjeldahl flasks containing 50 cc. C.P. sulfuric acid. When it was evident that some of the feces had been contaminated by urine, they were transferred, before placing in the Kjeldahl flasks, to a sheet of clean filter paper laid over screen wire and washed by a stream of hot, slightly acidified, nitrogen-free water from a wash bottle. In the digestion trials for dry matter, ether extract, and sugar, the feces were transferred to paraffined paper cups, in which they were dried for two to three hours at a temperature of 60° to 70°C. They were then placed in glass jars provided with rubber rings and glass covers and kept in a refrigerator until analyzed.

The methods of analysis prescribed by the Association of Official Agricultural Chemists were followed. Qualitative tests only for sugar were made, both Benedict's and Fehling's methods being employed.

#### DISCUSSION OF RESULTS

The apparent digestibility of the protein of fresh whole milk was found to be substantially higher than that of powdered whole milk. The mean of 47 coefficients previously reported (Neuens and Shaw, 14) is  $92.3 \pm .17$ , while the mean of 29 coefficients reported in this paper is  $90.9 \pm .20$ . The mean for the 76 determinations is  $91.8 \pm .14$ .

The range of the 26 coefficients of apparent digestibility of protein in powdered whole milk (Table I) is 84.4 to 90.9. The mean of the coefficients is  $87.4 \pm .22$ . But little difference was found in the coefficients for spray process milk and for roller process milk.

In a few cases the amounts of protein consumed individually by pair mates were widely different. It is believed, however, that a comparison of the records of pair mates is permissible, since the intake of a smaller or larger amount of protein than the average for the group seemed to have no influence upon digestion coefficients. For example, in the case of Pairs 301-302 and 303-304 (Table I, Period 2) the protein intake shows a rather

**TABLE I**  
**COMPARATIVE APPARENT DIGESTIBILITY OF THE TOTAL PROTEIN OF FRESH WHOLE MILK AND OF**  
**POWDERED WHOLE MILK**

Animal No.	Live weight (grams)	Protein in milk consumed (grams)	Coefficient of apparent digestibility	Animal No.	Live weight (grams)	Protein in milk consumed (grams)	Coefficient of apparent digestibility
Period 1							
Powdered whole milk (spray process)				Fresh whole milk			
291	294	29.1	89.4	292	271	26.7	91.2
293	208	19.7	89.1	294	211	23.3	91.1
295	216	21.9	84.6	296	195	20.4	91.2
297	217	25.0	*	298	206	21.8	91.6
299	200	22.0	86.7	300	203	21.6	91.2
301	167	17.8	88.4	302	164	20.4	92.6
303	241	29.0	*	304	258	23.8	93.4
305	183	23.9	90.9	306	179	20.9	91.7
335	267	28.0	*	336	250	25.5	91.5
337	240	22.0	89.1	338	223	23.3	92.5
Period 2							
Fresh whole milk				Powdered whole milk (spray process)			
291	321	25.5	90.9	292	271	25.7	85.8
293	223	23.5	92.2	294	205	16.7	86.6
295	228	22.0	91.1	296	204	23.8	88.0
297	228	21.0	90.2	298	209	22.6	86.6
299	203	19.9	91.3	300	197	16.3	87.1
301	177	20.2	90.9	302	165	14.9	88.1
303	277	23.8	92.9	304	310	31.5	88.6
305	192	22.5	91.0	306	188	20.0	85.7
335	315	25.5	*	336	272	27.0	87.6
337	270	25.3	92.4	338	247	22.6	87.6
Period 3							
Fresh whole milk				Powdered whole milk (roller process)			
363	165	18.7	88.4	364	162	21.9	85.8
365	177	21.2	88.2	366	158	19.6	85.0
367	201	18.7	89.7	368	218	27.3	88.4
369	140	11.7	88.4	370	156	18.0	87.9
371	204	21.2	89.9	372	218	25.4	90.1
373	146	14.2	92.6	374	159	23.0	86.1
375	170	16.7	89.4	376	156	19.6	85.8
377	177	16.3	85.8	378	187	17.2	84.4
379	193	15.6	91.4	380	218	27.3	*
381	194	18.7	90.6	382	206	22.3	89.0

\* Determination of protein in feces lost.

Note: The records of pair mates are shown on the same line. Thus Nos. 291 and 292 are pair mates, etc.

wide difference but the coefficients are much the same as those for the other animals of their groups.

The differences shown by pair mates in the apparent digestibility of protein indicate with a high degree of probability that there is a true difference in the nutritive qualities of the two kinds of milk. An average difference as great as that found with the seven pairs of animals fed fresh whole milk and powdered whole milk in Period 1 (Table I) might be expected to occur by chance but once in 470 trials conducted in the same manner. Likewise, an average difference as great as that found with the nine pairs of animals used in Period 2 (Table I) might be caused by chance not more than once in 10,000 repetitions of the experiment. For Period 3, the probability is once in 220 trials. These calculations of probabilities follow the method of Student (21).

The reversal trials (Table I, Periods 1 and 2) give further weight to the evidence that the apparent digestibility of the protein of fresh whole milk is higher than that of powdered whole milk. Not only is the coefficient secured with each animal fed fresh whole milk higher than that of its pair mate fed powdered whole milk, but the apparent digestibility of protein by each animal was more complete when fed fresh whole milk than when the same animal was fed powdered whole milk.

The authors (Nevens and Shaw, (14) previously reported that 45 coefficients of apparent digestibility of the protein of evaporated milk had a mean of  $88.4 \pm .25$ . The rather close agreement of that result with the result here given for powdered whole milk ( $87.4 \pm .22$ ) is interesting in view of the fact that evaporation is usually one of the procedures in the process of drying milk.

The fat of both fresh whole milk and powdered whole milk was found to be highly digestible (Table II). The mean of 30 coefficients for fresh whole milk is  $98.7 \pm .06$ , while for an equal number of coefficients for powdered whole milk it is  $98.7 \pm .08$ . There were no significant differences in the apparent digestibility of the fat of the two brands of powdered milk.

In view of the likelihood that ether soluble substances other than fat are normally present in the feces, it seems reasonable to assume that the fat of milk is almost completely, if not entirely, digestible. It is often claimed that manufacturing processes, such as homogenization, which reduce the fat globules to smaller size, increase the digestibility of the fat. The results reported in this and the previous paper (Nevens and Shaw, (14) raise a question regarding the soundness of such claims, since the digestibility of the fat of fresh whole milk is so nearly complete that there is but little possibility of its being increased.



TABLE II  
COMPARATIVE APPARENT DIGESTIBILITY OF THE FAT AND TOTAL SOLIDS OF FRESH WHOLE MILK AND OF POWDERED WHOLE MILK

Animal No.	Live weight grams	Fat in milk consumed grams	Total solids in milk consumed grams	Coefficient of ap- parent digestibility		Animal No.	Live weight grams	Fat in milk consumed grams	Total solids in milk consumed grams	Coefficient of ap- parent digestibility	
				Fat	T. solids					Fat	T. solids
Period 4											
Fresh whole milk						Powdered whole milk (spray process)					
291	346	21.0	84.3	99.7	92.1	292	299	22.4	76.6	99.2	90.7
293	233	19.2	77.3	99.5	92.2	294	210	19.5	66.9	99.7	91.3
295	241	19.7	79.2	99.6	93.7	296	210	12.2	41.9	99.5	89.1
297	240	18.3	73.6	99.2	92.5	298	223	18.7	64.0	99.5	89.6
299	217	17.6	70.8	99.0	92.7	300	203	19.2	65.7	99.5	90.9
301	184	13.7	55.0	99.5	92.7	302	170	16.1	55.1	99.5	92.1
303	305	19.2	77.3	98.0	92.4	304	358	33.7	115.4	99.2	91.5
305	205	16.6	66.9	99.4	92.7	306	203	20.1	68.9	99.5	91.1
335	348	24.4	98.2	98.8	92.7	336	303	25.9	88.7	99.3	92.1
337	303	19.3	77.4	98.8	91.4	338	280	23.3	79.6	99.2	91.0

Period 5

Fresh whole milk					Powdered whole milk (spray process)						
363	124	16.7	63.7	98.3	92.1	364	116	20.3	72.4	97.7	90.1
365	130	17.5	66.6	97.9	92.1	366	115	23.0	82.2	97.8	89.2
367	140	17.7	67.4	98.2	91.5	368	130	20.4	72.7	98.0	93.5
369	105	13.0	49.5	98.1	93.0	370	109	19.2	68.4	97.9	90.8
371	129	16.8	64.0	98.4	92.6	372	118	22.0	78.4	97.5	90.0
373	114	13.9	53.2	98.1	91.9	374	116	18.8	67.2	98.1	90.8
375	112	13.9	52.9	98.4	92.8	376	105	16.9	60.1	97.7	90.2
377	138	15.6	59.4	98.9	92.0	378	131	22.4	79.9	98.5	91.7
379	134	14.2	54.1	98.7	92.3	380	138	23.4	83.3	97.9	88.8
381	131	15.7	59.8	98.4	90.8	382	125	17.5	62.4	98.1	92.5

Period 6

Fresh whole milk					Powdered whole milk (roller process)						
363	178	22.6	89.8	98.9	91.6	364	172	24.2	84.7	99.0	91.1
365	190	22.9	91.1	98.5	91.0	366	173	23.5	82.1	98.8	91.2
367	214	19.1	75.9	98.6	91.2	368	245	26.1	91.3	99.1	92.5
369	157	15.3	60.9	98.1	91.8	370	164	19.7	69.0	98.7	91.0
371	223	21.6	85.9	98.7	91.8	372	253	29.7	103.7	98.3	89.4
373	162	18.3	72.8	98.0	90.9	374	176	26.2	91.7	99.3	91.3
375	195	19.9	79.1	98.7	93.1	376	172	25.4	88.8	98.7	91.0
377	199	21.2	84.3	98.9	90.9	378	196	19.6	68.7	99.1	91.8
379	222	21.3	84.8	98.4	92.0	380	238	29.9	104.6	98.8	90.3
381	211	21.5	85.5	99.0	90.6	382	240	27.3	95.5	98.8	90.8

Careful tests by both Benedict's and Fehling's methods have failed to reveal the presence of sugar in the feces of animals fed either fresh whole milk or powdered whole milk. Tests on individual collections of feces from 20 animals fed fresh whole milk, 10 animals fed spray process powdered whole milk, and 10 animals fed roller process powdered whole milk were all negative for sugar. It is believed, therefore, that the sugar of fresh whole milk and of powdered whole milk is completely digestible.

Assuming that the digestibility of the protein in the different kinds of milk varies, that the fat is more than 98 per cent digestible and that the sugar is completely digestible, then it is to be expected that the digestibility of the total solids of a particular sample of milk is influenced largely by the digestibility of its protein. This expectation is in harmony with the experimental results (Table II).

Thirty coefficients of apparent digestibility of the total solids of fresh whole milk have a mean of  $92.0 \pm .09$ , while the mean of an equal number of coefficients for powdered whole milk is  $90.9 \pm .13$ . It is evident that these coefficients are slightly higher in each case than those for protein of the respective kinds of milk, and that fresh whole milk is superior to powdered whole milk in digestibility of total solids. These coefficients for total solids lie between the coefficients for protein and for the other solids, but are not quite so high as would be expected by calculation. They tend, however, to substantiate the results found for protein, which indicate lower digestibility values for the powdered milk.

Many references are found in the literature to the "ease of digestibility" of certain kinds of milk, and the term "more digestible" is also commonly used. Many of these terms are deduced from the observations of physicians in cases in which they have found that one kind of milk agrees with the patient, while another kind causes more or less digestive disturbance or is unsatisfactory for some reason. There are many statements to the effect that evaporated milk and dried milk are "more digestible" than fresh raw milk.

The authors believe that their work helps to clarify the situation which now exists with respect to the term digestibility as applied to milk. Claims that homogenization, evaporation, or drying, or a combination of these factors, makes the protein and fat of milk more *completely* digestible lack the support of adequate experimental evidence obtained in actual feeding tests. The authors' findings, however, do not preclude the possibility that manufacturing processes such as those just mentioned may affect the time required for the digestion of the protein and fat, or that they may make the milk more readily tolerated by some individuals. In view of the differ-

ences between individuals with respect to their tolerance for certain foods, it is entirely possible that in some cases the *total* nutritive effect of evaporated milk and of powdered milk may be more favorable than that of fresh whole milk.

#### SUMMARY AND CONCLUSIONS

The apparent digestibilities of the total protein, fat, sugar, and total solids of fresh whole milk, spray process powdered whole milk, and roller process powdered whole milk were studied in feeding tests with albino rats. No significant differences in the apparent digestibility of the two kinds of powdered milk were found.

The apparent digestibility of the protein of fresh whole milk was significantly higher than that of powdered whole milk. It was shown in reversal trials that the higher coefficients for the fresh whole milk were not related to the individuality of the animals but that each animal digested the protein of fresh whole milk more completely than that of powdered whole milk.

The fat of both fresh whole milk and powdered whole milk was found to be about 99 per cent digestible, while the sugar of both kinds of milk was completely digestible.

Coefficients of apparent digestibility of total solids lay between those for protein and those for fat and were higher in the case of fresh whole milk. This substantiates to some extent the findings with respect to the apparent digestibility of protein.

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## FACTORS WHICH DETERMINE RENAL WEIGHT

### XIII. THE HEAT PRODUCTION OF THE RAT AS VARIED BY THYROID ADMINISTRATION

By

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THE administration of an active thyroid preparation has been shown (MacKay and MacKay, 7) to lead to an increase in the weight of the kidneys greater than the increase in protein intake incident to the increased food consumption could account for. Experiments have been performed to examine the relative influence of the active thyroid material upon the kidney weight and the heat production of the organism.

Desiccated thyroid<sup>1</sup> was used in this study. In the first experiment the diet described in Table I was used. Four cages of male albino rats were fed from 90 to 195 days of age. One group (B1) received no thyroid. The

TABLE I  
CONTROL DIET

Commercial casein.....	20
Cornstarch.....	36
Dried powdered brewer's yeast (Harris).....	10
Salt mixture (Osborne and Mendel).....	4
Cod liver oil.....	10
Lard.....	20

other groups received (B2) 0.12 per cent, (B3) 0.18 per cent and (B4) 0.30 per cent respectively. At the end of the 105 days the rats were starved for 24 hours and their basal oxygen consumption determined under chlorotone anesthesia using the method described by Dock (3). A tracheal cannula was closely connected to a 6 cm. glass tube containing soda lime for carbon dioxide absorption, which in turn led into a small bottle filled with strongly alkaline solution. A thermometer, the tube from the oxygen tank and the spirometer, a 20 cc. Luer syringe fitted with a kerosene lubricated Bakelite float, also led into this. The temperature of the system varied inappreciably during the required four or more measurements of the oxygen utilization rate. The environmental temperature was maintained at 29°C. The

<sup>1</sup> Wilson Laboratories, U. S. P.

rats were then sacrificed and their kidneys weighed with precautions described before (MacKay and MacKay, 5). The data obtained are presented in Table II. The administration of thyroid material naturally increased the caloric intake and hence the amount of protein ingested. This of itself would increase the weight of the kidneys (MacKay, MacKay, and Addis,

TABLE II

Per 100 sq. cm. body surface									
No.	Group	Body weight gm.	Body surface sq. cm.	Kidney weight mgm.	Food intake gm./day	Protein intake gm./day	Kidney weight due to protein mgm.	Corrected kidney weight mgm.	Oxygen consumption cc./min.
1	B1	278	483	165	2.09	0.46	25	140	0.87
2	B1	274	478	168	2.09	0.46	25	143	0.79
3	B1	302	511	170	2.09	0.46	25	145	0.85
4	B1	282	488	177	2.09	0.46	25	152	0.86
5	B1	287	494	193	2.09	0.46	25	168	0.83
6	B2	246	445	209	3.17	0.69	38	171	1.33
7	B2	227	422	212	3.17	0.69	38	174	1.36
8	B2	230	426	231	3.17	0.69	38	193	1.31
9	B2	220	413	233	3.17	0.69	38	195	1.78
10	B3	167	344	253	3.78	0.82	45	208	1.74
11	B3	218	411	266	3.78	0.82	45	221	1.75
12	B3	212	404	282	3.78	0.82	45	237	1.88
13	B4	198	386	294	3.97	0.87	48	246	2.30
14	B4	193	379	277	3.97	0.87	48	229	2.06
15	B4	178	359	288	3.97	0.87	48	240	2.48

9). When the protein is casein, the relationship between the protein ingested and the kidney weight may be expressed (MacKay and MacKay, 6) as *protein intake* (grams per 100 sq. cm. body surface per day) =  $0.0183$  *renal weight* (milligrams per 100 sq. cm. body surface) - 2.75 or the kidney weight is dependent upon the protein as *renal weight* = *protein intake*  $\times 55$ . Our results have all been corrected for the effect of ingested protein in order to make their comparison with the oxygen consumption more simple. As heretofore, all kidney weights are the average figure for the two kidneys. The kidney weight corrections made necessary by the fluctuation in the protein intake are very different in the four groups and another experiment was carried out with the same diet containing 0 (A1), 0.06 (A2), 0.12 (A3), 0.18 (A4) and 0.24 (A5) per cent desiccated thyroid respectively, identical in every way with the first except that 15 days before the

end of the experiment some of the casein in the various diets was replaced with cornstarch. The data for this group comprise Table III. The protein corrections are somewhat more uniform.

TABLE III

Per 100 sq. cm. body surface									
No.	Group	Body weight gm.	Body surface sq. cm.	Kidney weight per 100 cm <sup>2</sup> mgm.	Food intake gm./day	Protein intake gm./day	Kidney weight due to protein mgm.	Corrected kidney weight mgm.	Oxygen consumption cc./min.
1	A1	286	492	160	1.77	0.43	24	136	0.83
2	A1	284	490	162	1.77	0.43	24	138	0.71
3	A1	308	518	175	1.77	0.43	24	151	0.85
4	A1	281	487	177	1.77	0.43	24	153	0.78
5	A1	288	495	192	1.77	0.43	24	168	0.89
6	A2	246	445	208	2.85	0.50	28	180	1.19
7	A2	270	473	210	2.85	0.50	28	182	1.27
8	A2	246	445	214	2.85	0.50	28	186	1.19
9	A2	230	426	215	2.85	0.50	28	187	1.22
10	A2	240	437	216	2.85	0.50	28	188	1.44
11	A3	250	450	191	2.98	0.48	27	164	1.62
12	A3	218	411	216	2.98	0.48	27	189	1.58
13	A3	195	382	225	2.98	0.48	27	198	1.93
14	A3	235	432	249	2.98	0.48	27	222	1.62
15	A3	210	402	254	2.98	0.48	27	227	1.82
16	A4	184	368	262	4.16	0.57	31	231	1.85
17	A4	188	373	273	4.16	0.57	31	242	2.17
18	A4	190	375	292	4.16	0.57	31	261	2.16
19	A5	207	397	260	4.53	0.62	34	226	1.94
20	A5	198	386	269	4.53	0.62	34	235	2.15
21	A5	204	394	291	4.53	0.62	34	257	2.30
22	A5	212	404	327	4.53	0.62	34	283	2.44

The data for both experiments have been plotted in Figure 1. Body surface was calculated by the formula devised by Carman and Mitchell (2). There is a definite, probably linear, relation between the kidney weight and the basal oxygen consumption of the organism. When we measure the total heat production of the organism by the food intake, a reasonable procedure since the animals were neither gaining nor losing weight, there is also a linear relationship (Fig. 2) between this figure and the kidney weight. This indicates that the thyroid material raised the total metabolism of our rats solely through its effect upon basal heat production.



The linear relation between the basal oxygen consumption of the organism and the kidney weight is very similar to that found for heart weight (Smith and MacKay, 11). In the case of heart weight it was possible to offer a reasonable explanation of the relationship upon the basis of the probable work performed by the organ. With the kidney this is not so simple. The excretion of the products of exogenous protein catabolism is

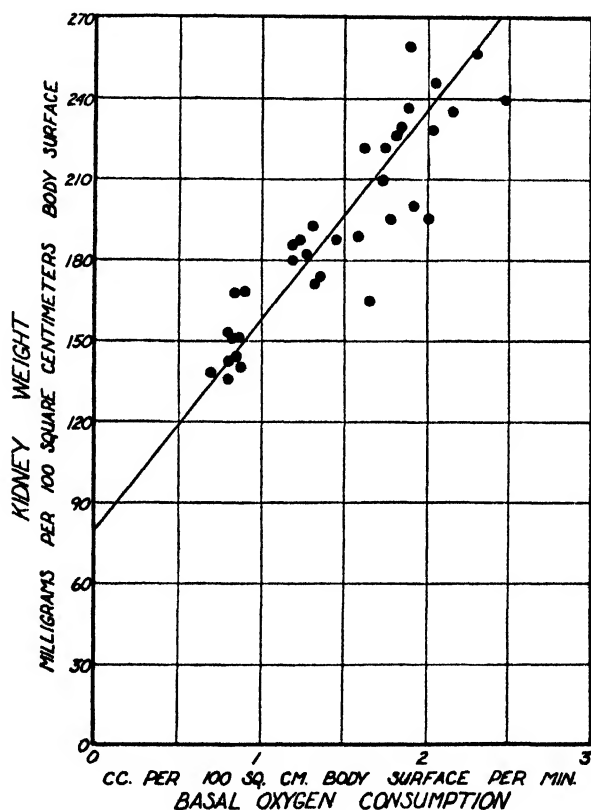


FIG. 1.

obviously part of the work of the kidney. The effect of this work on the kidney has been subtracted before considering the relation to the basal oxygen consumption in Figure 1. There is the possibility that the renal weight as varied by thyroid is governed by the endogenous protein catabolism which is increased (MacKay and Cockrill, 4) by thyroid. However an increase in the endogenous protein catabolism produced by starvation or dehydration is not followed by an increase in renal weight (MacKay and Cockrill, 4) and we are of the opinion that the relation between kidney

weight and the endogenous protein metabolism, when both are varied by thyroid, is not causal but due to their dependence upon the same factor, namely, the increased level of the basal metabolism.

Phosphate excretion is increased by thyroid (Aub, Bauer, Heath, and Ropes, 1) and an increased phosphate excretion due to feeding inorganic phosphates produces an increase in the weight of the kidneys (MacKay,

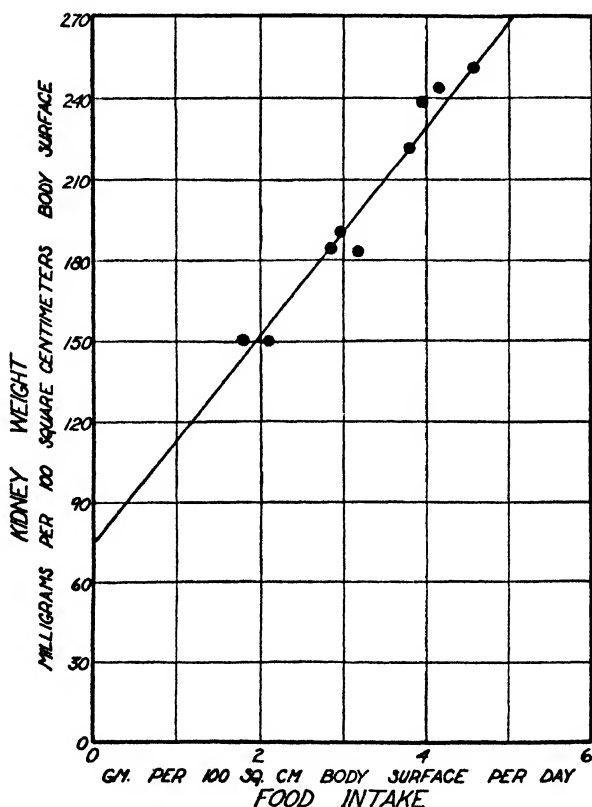


FIG. 2.

MacKay, and Addis, 8). It seems very unlikely that an increase in the urinary phosphate excretion contributes a significant part of the renal weight increase due to thyroid for the relation between renal weight and the basal metabolism is very good while between urinary phosphate excretion and the basal metabolism it is only roughly positive. Furthermore, kidneys of the weight found after thyroid administration, if due to phosphate, would show definite pathology (MacKay and Oliver, 10) and in the present experiments all of the kidneys were normal.

In the present state of our knowledge of the effect of thyroid upon the weight of the kidneys we are unable to offer any reasonable explanation for the increase in renal weight following thyroid administration and the relationship between the renal weight and the heat production of the organism when this is varied by thyroid.

### SUMMARY

In albino rats receiving thyroid there is a linear relationship between renal weight and caloric requirements of the animal as measured by oxygen consumption under basal conditions (fasting and chloretone anesthesia) or under conditions of normal activity (measured by daily food intake).

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# FACTORS WHICH DETERMINE RENAL WEIGHT

## XIV. THE RELATIVE INFLUENCE OF AMINO, UREA AND PROTEIN NITROGEN IN THE DIET

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IT has been shown (MacKay, MacKay, and Addis, 2) that urea nitrogen in the diet has less effect upon renal weight than a corresponding amount of nitrogen in the form of casein. Although the ultimate fate of most of the protein nitrogen is urea nitrogen, it actually reaches the body as amino nitrogen. The influence upon the weight of the kidneys of young male albino rats of amino, urea, and protein nitrogen has been compared.

The protein nitrogen was fed as casein and the amino nitrogen as glycine and glutamic acid. The cost of the dietary ingredients precluded any large number of rats in the diet groups but we believe that the great care which was exercised in all measurements compensates in large degree for the small number of animals. In other experiments on renal weight effect in young rats the animals were fed from 26 to 70 days of age. We have found that diets regulating renal weight reach their maximum effect within 10 days. In this experiment the rats were fed from 60 to 70 days of age. During the last 9 days of this period food consumption and the excretion of nitrogen in the urine were measured. The averages for this period form the basis of our data. In any experimental series a litter mate was placed upon each diet so that a series of a group on the control diets and one upon each of the other four diets represents several sets of 5 litter mates each.

The diets are described in Table I. They are identical in caloric value and, within a given group, in nitrogen content. The control (C) diet contained 3.75 per cent nitrogen, 2.73 per cent of which was in the form of casein. All of the "A" diets contained 2.38 per cent more nitrogen than the control diet or 6.13 per cent. The "B" diets contained twice as much additional nitrogen as the "A" diets giving a total of 8.52 per cent. The casein used was a specially purified product marketed by the Harris Laboratories (Tuckahoe, N. Y.). Powdered brewers yeast came from the same source. Osborne and Mendel's (5) salt mixture was used. The urea, glutamic acid, and glycine were recrystallized from C. P. products.

TABLE I  
COMPOSITION OF DIETS

Diet	C	1A	2A	3A	4A	1B	2B	3B	4B
Casein	20.	37.5	20.	20.	20.	54.9	20.	20.	20.
Yeast	10.	10.	10.	10.	10.	10.	10.	10.	10.
Cod liver oil	10.	10.	10.	10.	10.	10.	10.	10.	10.
Salt mixt.	4.	4.	4.	4.	4.	4.	4.	4.	4.
Cornstarch	50.	32.5	44.8	25.	37.2	15.1	39.7		24.4
Lard	6.	6.	6.	6.	6.	6.	6.	6.	6.
Urea			5.2				10.3		
Glutamic acid				25.				50.	
Glycine					12.8				25.6

The rats upon all of the diets did reasonably well with the exception of those receiving the higher concentration of glutamic acid. The animals disliked this food and their intake was insufficient for maintenance. This does not however impair the value of our average figures. After ten days

TABLE II  
ORGAN WEIGHTS AND RELATIONSHIPS

Diet	No. Rats in group	Gross Body Weight		Length mm.	Body Surface sq. cm.	Per 100 square centimeters per day				
		Initial gm.	Death gm.			Heart Weight mgm.	Food Intake per day gm.	Protein Intake per day gm.	Nitrogen Intake as protein** per day gm.	Nitrogen Excretion per day mgm.
C	3	112	165	188	331	187	3.78	0.737	0.790	70
1A	3	115	156	187	322	184	3.51	1.210	1.252	138
2A	3	114	154	186	323	185	2.64	0.515	0.943	185
3A	3	113	131	177	282	177	3.17	0.618	1.132	117
4A	3	114	152	183	309	188	3.55	0.693	1.267	179
C	3	105	140	179	292	197	3.36	0.656	0.720	90
1A	3	106	147	177	298	203	3.45	1.190	1.233	181
2A	3	106	142	176	292	198	4.02	0.784	1.438	219
3A	3	108	131	174	268	200	3.36	0.656	1.201	160
4A	3	109	132	175	273	206	3.34	0.652	1.215	195
C	4	122	160	183	321	173	3.48	0.680	0.731	86
1B	4	121	136	177	293	170	3.22	1.585	1.635	181
2B	4	121	153	182	305	172	3.56	0.695	1.810	261
3B	4	120	111	172	244	169	1.78	0.348	0.905	115
4B	4	120	133	176	271	170	2.68	0.524	1.362	190

\*\* The yeast nitrogen not included as protein under the observed protein intake is excluded here.

on the diets the rats were starved for 12 hours and then killed. Anatomical measurements were made by methods described elsewhere (MacKay and MacKay, 1). The results are presented in Table II.

The dietary protein has a marked effect upon the renal weight. When this protein is casein this weight may be expressed (MacKay and MacKay, 3) as: *Renal Weight* (mgm. per 100 sq. cm. body surface) =  $(\text{Protein Intake (gm. per 100 sq. cm. B. S.)} + 2.75) \div .0183$  or the renal weight due to protein may be expressed: *Renal Weight* = *Protein Intake*  $\times 55$ . These formulae were used for treating the data in Tables II and III. Since the kidney weight calculated from the actual protein intake (Table III) is somewhat higher for the high protein diets, a great deal higher for the urea diet and much less for the amino acid diets than the observed figures, we confirm our earlier result that urea has less effect on renal weight than protein nitrogen and conclude that amino nitrogen both in the form of glutamic acid and glycine has a definite influence upon renal weight. From the

TABLE II  
ORGAN WEIGHTS AND RELATIONSHIPS

Per 100 square centimeters per day							Heart: kidney ratio	Differ- ence per cent	Per cent H <sub>2</sub> O in Kidney
Kidney* Weight actual mgm.	K. Wt. calc. from protein intake mgm.	Differ- ence per cent	K. Wt. calc. from N. intake as pro- tein mgm.	Differ- ence per cent	K. Wt. calc. from N. excre. as pro- tein mgm.	Differ- ence per cent			
195	191	-2	193	-1	174	-11	1.05	100	75.4
200	216	8	218	9	197	-1	1.09	105	74.3
196	179	-9	202	3	214	9	1.05	101	74.6
186	184	-1	212	14	190	2	1.06	101	74.5
204	188	-8	219	2	212	4	1.08	103	74.0
193	186	-4	189	1	181	-6	1.02	100	76.4
223	215	-4	217	1	212	-5	1.10	108	76.4
206	193	-6	229	19	225	9	1.04	103	75.9
193	186	-4	216	16	205	6	0.97	95	74.7
219	186	-16	218		217	-1	1.06	104	75.6
178	186	4	190	6	180	1	1.03	100	76.6
209	237	12	240	15	212	1	1.22	117	76.3
193	188	-3	249	29	239	22	1.13	110	75.6
212	169	-23	200	-6	190	10	1.26	123	75.6
231	179	-22	224	-3	215	-7	1.36	132	75.9

\* Average weight of the two kidneys as used throughout this study.

TABLE III  
KIDNEY WEIGHT DEPENDENT ON PROTEIN INTAKE\*  
(Protein Intake in grams  $\times 55$  = Kidney Weight in Milligrams)

Diet	Observed**				Calculated from the actual protein intake				
	A1	A2	B	Average	A1	A2	B	Average	Per cent dev.
C	45	43	28	39	41	36	37	38	-3
1	50	73	59	61	60	60	80	66	+14
2	46	56	43	48	29	43	38	36	+28
3	36	43	62	47	34	36	19	29	-35
4	54	69	81	68	38	35	29	34	-47

Calculated from the Nitrogen intake as protein					Calculated from the Nitrogen excretion as ingested protein				
A1	A2	B	Average	Per cent dev.	A1	A2	B	Average	Per cent dev.†
43	39	40	40	+3	24	31	30	28	0
68	67	90	75	+23	42	62	62	55	+18
52	79	99	77	+106	63	75	90	76	+86
62	66	50	56	+19	40	55	40	44	+22
69	68	74	70	+3	62	67	65	64	+22

\* Mgms. per 100 sq. cm. body surface.

\*\* Determined as difference between 150 (required for zero protein intake) and actual figure for renal weight in mgms. per 100 sq. cm. body surface.

† Adjusted to zero basis for control group.

average results of the relation of the renal weight calculated from the nitrogen intake considered as protein, and particularly from the nitrogen excretion in the urine considered as food protein, to the observed figures, we may conclude that nitrogen in the form of glutamic acid and glycine have practically the same influence upon renal weight as casein nitrogen and that the nitrogen of all of these are more effective in increasing the weight of the kidneys than urea nitrogen. It is interesting that none of the diets had a significant effect upon the water content of the kidneys.

When the basal metabolic rate is raised by the administration of thyroid the weight of the kidneys is increased (MacKay, Smith and Closs, 4). The possibility existed that casein nitrogen had a greater influence on renal weight than urea nitrogen because casein raises the metabolic rate while

urea does not. Since glutamic acid, which is without influence upon the metabolic rate, and glycine which raises it, are similar in their effect upon renal weight, we must discard this hypothesis.

#### SUMMARY

Amino nitrogen in the form of glutamic acid and glycine have practically the same influence on renal weight as protein nitrogen in the form of casein. Urea nitrogen is less effective than amino or protein nitrogen.

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## IS FLUORINE AN INDISPENSABLE ELEMENT IN THE DIET?\*

By

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FLUORINE is the most active, chemically, of the elements. It is widely distributed in soils, rocks, and waters, being estimated to constitute 0.1 per cent of the first half mile of the earth's crust, land and sea. It is placed twentieth in order of abundance among the elements in the surface half mile of the earth. In certain spring and well waters it is so abundant as to cause mottling of the enamel of the teeth of persons drinking such waters while the enamel is forming (1). Fluorine is apparently universally present in plant and animal tissues. The question is still unanswered as to whether the element plays a physiological rôle or is present in the tissues as an accidental constituent because it is ingested in all foods.

Morichini (2) in 1802 first found fluorine in biological material when analyzing fossil elephant molars. Since that time many investigators have examined numerous animal and plant tissues for fluorine. Interest has centered mainly in the fluorine content of teeth and bones. Trebitsch (3) gives a review of the analyses up to 1927. The soft tissues are poor in fluorine, but bones, enamel, and dentine are relatively rich in it.

McCollum and co-workers (4) first described the effects of feeding to rats a diet containing 226 parts per million of sodium fluoride. This amount is excessive in comparison with the amounts generally consumed by humans or animals in food and water. Rats fed a diet otherwise satisfactory but containing excessive fluorine showed defective dentition. Christiani and Gautier (5) report that fluorine tends to accumulate in the bones of guinea pigs producing a condition resembling osteomalacia. Begara (6) reported that feeding excessive amounts of fluorine retarded calcification of the osseous tissues and produced dark bands in the incisors. Smith has extended these investigations to human subjects (1). The present paper is a report of experiments to determine whether satisfactory nutrition can be

\* The data reported in this paper are taken from the dissertation of Dr. George R. Sharpless, submitted in partial fulfillment of the requirements for the degree of Doctor of Science in Hygiene.

secured during growth and reproduction with diets depleted as far as possible in fluorine.

#### METHOD OF DETERMINING FLUORINE

The quantitative methods for the determination of fluorine were studied critically and a combination of the volatilization procedure of Reynolds, Ross and Jacobs (7) and of Wagner and Ross (8) and the colorimetric method of Merwin (9) and Stieger (10) was adopted. Our procedure was based upon the formation and volatilization of silicon tetrafluoride when a substance to be analyzed is intimately mixed with fine silica and decomposed with concentrated sulfuric acid. The colorimetric method was based upon the bleaching action of fluorine on the yellow color of an oxidized titanium solution.

#### REAGENTS

*Concentrated  $H_2SO_4$ .*—The concentrated sulfuric acid used was prepared by boiling Baker's C.P. concentrated sulfuric acid a short time in order to remove any dissolved fluorine and  $SO_2$ . The  $SO_2$  interferes in the colorimetric determination only by necessitating the use of a larger quantity of hydrogen peroxide than is ordinarily used in the procedure. The acid is stored in a ground glass stoppered bottle to prevent absorption of moisture. It was found unnecessary to have the acid as concentrated as 98.5 per cent, as is advocated by Wagner and Ross (8). This point was tested by using a lower concentration of acid and also by using various amounts of calcium oxide in the sample. It was found that 90 per cent acid was just as effective as the 98.5 per cent in a corresponding temperature range up to  $200^\circ$ ; however, above  $200^\circ C$ . water is given off and upon condensing in the delivery tube interferes with the determination.

From the equation:  $CaO + H_2SO_4 = CaSO_4 + H_2O$  it can be calculated that nearly 3 grams of calcium oxide or its equivalent are necessary to react with five cubic centimeters of 98.5 per cent sulfuric acid to reduce it to 90.0 per cent.

*Silica.*—The silica used was Arthur H. Thomas's commercial silicic acid powder. Before use the silicic acid was boiled with concentrated sulfuric acid to remove any fluorine that might be present.

*Standard titanium solution.*—The standard used is empirical. A standardized twenty per cent solution of titanous chloride was diluted, 2.5 cc. to one liter, in 3 per cent sulfuric acid. The acid is necessary to prevent hydrolysis of the titanous chloride which takes place readily in a neutral or only slightly acid solution. A stronger acid (5–10 per cent sulfuric) will cause a darker yellow color upon addition of hydrogen peroxide but it is

not bleached to as great an extent by fluorine. This method of using a titanous solution to make up the standard is much simpler than the method of dissolving titanium dioxide as described by Stieger (10). Stieger recommends the use of a titanium solution containing 0.001 gm. titanous oxide per cubic centimeter. The above solution contains the equivalent of 0.000258 gm. titanous oxide per cubic centimeter which, when diluted as described in the procedure, gives a concentration equivalent to 0.0000516 gm. titanium oxide per cubic centimeter as the final dilution.

*Hydrogen peroxide.*—The hydrogen peroxide used was Baker's Analyzed C.P. 3 per cent. It was found to contain a small amount of fluorine but the amount needed for the oxidation was so small that the fluorine present was treated as a constant factor and thus played no rôle in the determination. The C.P. 3 per cent solution was diluted 1–5 before use in order to reduce the error in the measurement of small quantities of the liquid.

#### APPARATUS

The apparatus required for the volatilization process consists of the following individual pieces assembled in series:

(a) Two gas-washing bottles containing concentrated sulfuric acid to remove moisture from the air which is drawn through the apparatus during the determination.

(b) A cylinder loosely packed with glass wool and phosphorous pentoxide, also for removing moisture.

(c) A reaction tube for digesting the sample with concentrated sulfuric acid. This tube is an ordinary test tube with a side arm delivery tube. Air is introduced through a piece of glass tubing which runs down the center of the tube to the bottom, being led in through a rubber stopper. By using a T tube the acid is also led in through this same tube from a dropping funnel without disturbing any part of the apparatus. The air delivery tube is slightly bell shaped at the bottom so as to give larger bubbles and thus keep the contents in the rounded bottom portion of the reaction tube well agitated. (d) A furnace, with rheostat, for heating the reaction tube. A type 84, Eimer and Amend multiple unit electric furnace was used. (e) An absorption tube which consists of a small test tube fitted with an inlet tube which has been drawn out to a large capillary. The outlet tube is connected with a water aspirator through a pressure regulating vessel containing mercury. (f) The colorimeter used was a Leitz, Bürker type. The advantage of this instrument in this case is that two cubic centimeters of solution are sufficient for a determination.

## ASHING

The material to be tested for fluorine by the method used must, if it contains organic matter, be ashed by a suitable method. Fluorine is easily volatilized and thereby lost, so that extreme care must be exercised during this part of the procedure. It was found that there are three major potential sources of error in ashing. They are (a) high temperature, (b) acid ash, and (c) moisture in the sample when it is placed in the furnace.

The treatment of the sample before ashing depends entirely upon the nature of the substance. Bones and teeth were dried and ashed directly. Biological materials which gave an acid ash (usually containing a large amount of phosphorus) were mixed with calcium acetate if only slightly acid and with sodium hydroxide and calcium acetate if they gave a very acid ash, e.g. such as casein. The calcium added serves to make the ash alkaline and also acts as a catalyst in the combustion of the carbon. A number of other metals, cerium, lanthanum and thorium were tried as catalysts, as is advocated by Walters (11) in the ashing of flours, but were found to be inferior to calcium under the conditions necessary in this determination. The sodium hydroxide and calcium acetate were added to the sample in solution to insure a complete and uniform distribution of these two substances throughout the sample. The sample was then dried in an oven at about 80°C. before being ashed. It was found that if drying were not complete before the sample was placed in the muffle furnace, fluorine added as sodium fluoride was lost. If plant materials or any material containing boron in the form of borax or boric acid and probably other boron compounds are to be tested for fluorine by the volatilization of silicon tetrafluoride, it is necessary to add sodium carbonate to the sample before ashing to prevent the formation of a non-volatile boron fluoride compound.

Ashing was carried out in porcelain dishes placed in a muffle furnace at a temperature not exceeding a dull red heat (500°–600°C.). With some ashes a temperature much higher (700°–800°C.) will not volatilize an appreciable percentage of the fluorine but this is not true in all cases. Glazed porcelain dishes were found to be very satisfactory for ashing. The time necessary for complete ashing varies with the sample but if the ash is sufficiently alkaline the ashing will be complete in from two to three hours. Some difficulty in obtaining complete ashing is sometimes experienced with samples having a high phosphorus content. This difficulty, however, is easily overcome by increasing the amount of calcium added before ashing is begun.

The ash after being thoroughly mixed with a quantity of silica powder

is ready to be placed in the reaction tube. The quantity of silica will depend upon the amount of ash,—for one gram of ash 0.2 to 0.3 gm. of silica is sufficient; however, excess of silica does not seem to interfere in any way with the determination. The quantity advisable to use depends more on the total volume in the reaction tube than upon the size of the ash sample.

#### VOLATILIZATION OF FLUORINE AS SILICON TETRAFLUORIDE

The ash intimately mixed with silica as described above is placed in the reaction tube. It was found that if the reaction tube and delivery tube were dried at 80°–100°C. just previous to a determination, the percentage recovery was much greater and more constant (see Table I). Using sodium fluoride as the test material it was found that 70–90 per cent could be recovered (see Table I) if moisture were kept from all parts of the apparatus that came in contact with the silicon tetrafluoride gas.

TABLE I

COLORIMETRIC DETERMINATIONS TO COMPARE VALUES FOR FLUORINE FROM PURE NaF AND VOLATILIZED F

Amount F	Not volatilized	Volatilized in oven dried apparatus	Volatilized in air dry apparatus
Mg.	Reading	Reading	Reading
0	5.00	5.00	5.00
0.01	5.25	5.19	5.00
0.02	5.49	5.40	5.11
0.03	5.75	5.64	5.28
0.04	5.96	5.88	5.34
0.05	6.25	6.13	5.60
0.06	6.52	6.42	5.82
0.07	6.80	6.69	5.90
0.08	7.03	6.92	6.00
0.09	7.28	7.00	6.10
0.10	7.32	7.12	6.20

The reaction tube is then connected in place in the furnace and a stream of air drawn through the apparatus at such a rate that bubbles are formed at the end of the capillary delivery tube. Concentrated sulfuric acid—10 cc. for samples up to one gram and correspondingly more for larger samples—is then added through the air inlet tube, the rate of addition depending upon the nature of the sample. The temperature of the reaction tube is raised to 190°–200°C., kept there for one and one-half hours and then gradually lowered. The temperature is observed by means of a thermometer with the bulb placed about midway of the sulfuric acid column. It is

very necessary that the temperature be kept below 200°C., so that phosphoric acid will not be volatilized and carried over. The air drawn through the apparatus serves the following three purposes, (a) to sweep the silicon tetrafluoride formed over into the absorption tube, (b) to keep the sample and the silica constantly and thoroughly mixed and (c) as a mechanical agitator to prevent the trapping of minute bubbles of silicon tetrafluoride. The flow of air is regulated in such a way as to avoid the necessity of mechanical shaking of the reaction tube. The time necessary for a complete determination is 2-2 1/2 hours.

The absorption tube, as stated above, is small, two cubic centimeters of water being used as the absorbing medium. Sodium hydroxide was tried as the absorbing medium when a large amount of chlorine was present in the sample but was found to be unnecessary, as would be expected when the fact that fluorine replaces chlorine in solution is borne in mind. When the volatilization is complete the absorption tube is disconnected, the contents accurately measured and placed in a test tube. Sufficient water is then added to bring the total volume up to 3.5 cc. and it is ready for the colorimetric determination.

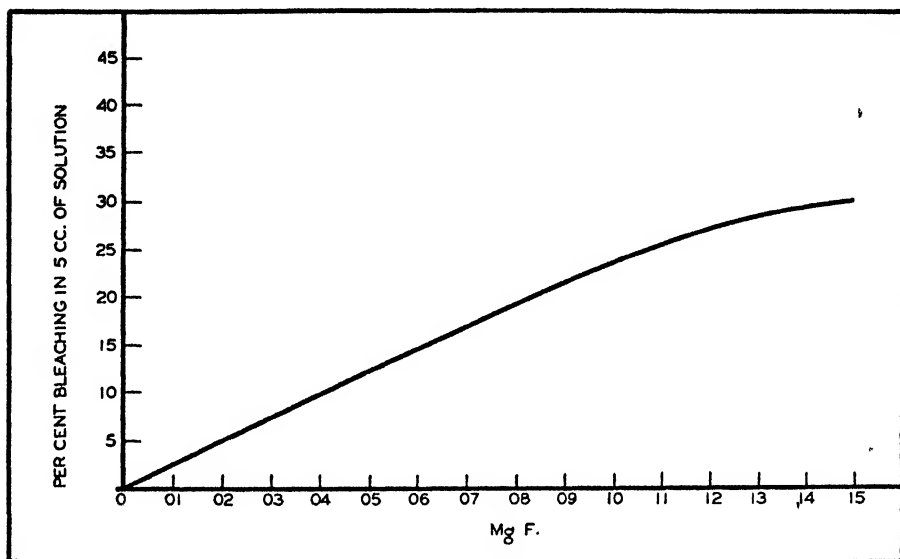
#### COLORIMETRIC DETERMINATION

The colorimetric determination, as stated above, depends upon the bleaching action of fluorine on the yellow color obtained when a solution containing titanium is oxidized by means of hydrogen peroxide. However, the per cent of color change, calculated as per cent bleaching, plotted against amount of fluorine present, does not follow a straight line after a 20 per cent bleach is obtained. Consequently a curve must be constructed as shown in Graph 1.

The Bürker colorimeter is constructed so that the standard solution always has an effective stratum thickness of 10 mm. Thus, a solution of the same strength as the standard would give a reading of 10, the scale being graduated in millimeters. However, since the nature of the construction of the instrument causes the range above the 10 mm. mark to be limited, and since all readings would be less than the standard, it was deemed advisable to use a solution in the "standard" cup of just one-half the strength of the solution used in the determination, thus giving a reading of 5.0 mm. for the standard solution. This relationship was used throughout the experiment. This method also provides a very simple way of determining the per cent of bleaching. It is obvious that a reading of 10 mm. would mean that half the color had disappeared or, in other words, it is a fifty per cent bleach; a reading of 6 mm. is a 10 per cent bleach, etc.

The concentration of the titanium solution chosen is such that it gives a light yellow color, yet concentrated enough to give a definite color in the colorimeter. This concentration was chosen for this reason rather than for any stoichiometrical relationship.

The procedure for the colorimetric determination is as follows: The solution containing the fluorine is made up to a volume of 3.5 cc. as stated



GRAPH 1.

above; 1 cc. of the standard titanium solution and 0.5 cc. of the 1-5 dilution of hydrogen peroxide is added, the total volume now being 5.0 cc. Part of this is used to rinse the cup and plunger of the colorimeter (1.5-2.0 cc. being sufficient for a determination). Ten individual readings are made and the average is taken as the value for that particular determination.

#### CALCULATION

In order to arrive at a correct figure for this fluorine process a few corrections must be applied. Stieger (10) called attention to the fact that the percentage bleaching is not proportional to the amount of fluorine. It was also found to be true in this work (see Graph 1). However, it will be seen from the graph that the per cent bleaching is proportional to the amount of fluorine present in the range from zero to twenty per cent. Thus, if the volume of solution is controlled so that the per cent bleaching will be less than 20 per cent it can be seen from the graph that the determination will,



in all probability, permit a more delicate determination. That is, the system produces a greater change in color per unit amount of fluorine up to a 20 per cent bleach. With this in mind, dilutions of the test solution were always made with the "5 mm. reading" standard when there was more than 20 per cent bleaching. However, it can readily be seen that there would be little advantage in diluting more than three times since—the calculation being made on a 5.0 cc. volume basis—the error introduced in the reading would be increased correspondingly. Consequently the size of sample was regulated so as never to contain more than 0.2 mg. of fluorine. In order to facilitate calculation, dilutions were always made in 5 cc. quantity.

According to Merwin (9), temperature plays a part in the intensity of color produced; however it is small and can be ignored in this case because all tests were made at room temperature, which varied but little.

The amount of fluorine present can be determined by calculating the per cent bleaching from the reading on the colorimeter and finding the amount of fluorine on Graph 1 that corresponds to that amount of bleaching. If the solution has been made up to ten cubic centimeters volume the answer is multiplied by two, etc.

It has been shown in Table I that the per cent recovery expected using pure sodium fluoride ranges from 70–90 per cent. Consequently, in the calculations each value was increased by one-fifth. That is, the observed value was considered to be 80 per cent of the fluorine actually present.

#### ACCURACY OF THE METHOD

That the method is accurate within reasonable limits of error for such small quantities of material involved is shown by the fact that a nice range of values can be obtained with differences of but 0.01 of a milligram and that a constant and large per cent of the fluorine can be recovered when NaF is added to a sample. Check results are also obtained when a sample having an unknown amount of fluorine is used. Table II shows some duplicate results on bone ash samples.

TABLE II  
FLUORINE DETERMINATIONS TO SHOW ABILITY TO DUPLICATE VALUES

Sample	Wt. Sample	Dilution	Reading mm.	Per cent F.
11	0.4172	5.0	5.53	0.0059
11	0.6779	5.0	5.80	0.0054
8	0.2218	10.0	6.89	0.0834
8	0.1438	5.0	7.04	0.0766

## PREPARATION OF LOW FLUORINE DIET

*Salts.*—The individual salts of McCollum's salt mixture No. 51, which includes the following salts in proportions shown, were tested for fluorine.

CaCO <sub>3</sub> .....	1.5
KCl.....	1.0
NaHCO <sub>3</sub> .....	.7
NaCl.....	.5
MgO.....	.2
Fe citrate.....	.5
KH <sub>2</sub> PO <sub>4</sub> .....	1.7

Each salt, with the exceptions of the phosphate and ferric citrate, was dissolved and tested directly by the colorimetric method. They were all tested by an etching method also. The potassium and sodium chlorides were also tested by the sensitive color reaction described by Boer (12). The ferric citrate was ashed and tested by the volatilization method. Baker's Analyzed Chemicals were used and, with the exception of the primary phosphate, were found by the above named methods to be free from fluorine. The phosphate which interferes with the bleaching action of the fluorine was tested by the volatilization procedure. Considerable fluorine was found in one sample; however, a sample from a different lot which was in the form of fine crystals, whereas the first lot consisted of large crystals, was found to give a negative test even when a special large reaction tube containing 10 gm. of the salt was used.

*Dextrin.*—The dextrin used in the stock ration in this laboratory was found to contain considerable fluorine. The starch from which the dextrin was made was also found to contain fluorine; however, the absolute amount was quite variable and some samples contained none. This suggested that the fluorine was held in a very loose way and that it might be possible to wash it out of the starch with acidified water. Such a method was tried and was found to remove the fluorine completely insofar as could be determined in the ash from 100 grams of material. The final procedure adopted was as follows: one-half pound of corn starch<sup>1</sup> per liter of three per cent HCl, by volume, was thoroughly churned by an electrically-driven stirrer for from two to three minutes, then the starch was allowed to settle, leaving a clear solution above. The clear supernatant solution was then drawn off and the process repeated three times with distilled water. The starch was dried in pyrex dishes in a warm air oven and then pulverized.

<sup>1</sup> Argo brand.

*Other constituents.*—The yeast used in this laboratory<sup>2</sup> was found to be free from fluorine insofar as could be determined. That is, less than 0.02 mg. per 100 gm. or less than one part per five million was present.

Butterfat, made by melting butter and filtering off the curd and water, also gave a negative test for fluorine in the ash from 100 grams.

Viosterol<sup>3</sup> was also tested and gave a negative test in the ash from ten cubic centimeters.

TABLE III  
COMPOSITION OF DIETS

Constituents	Test Diet	Control
Casein	18.0	18.0
Butterfat	8.0	8.0
Salts 51A*	6.5	6.5
Yeast	10.0	10.0
Starch	57.5	57.5
Viosterol	15 drops/kilo.	15 drops/kilo.
Sodium fluoride		0.003

\* Composition of salts 51A:

CaCO <sub>3</sub> .....	150.0 grams
KCl.....	100.0
NaCl.....	50.0
NaHCO <sub>3</sub> .....	70.0
Ferric citrate.....	50.0
KH <sub>2</sub> PO <sub>4</sub> .....	170.0
MgSO <sub>4</sub> .....	60.0

Casein as prepared in the laboratory by washing the commercial product with acetic acid and then with distilled water was found to contain a small amount of fluorine. However, a sample of the commercial product before washing gave a negative test for eight individual determinations using 50 grams for each. The material was also tested by adding 0.1 mg. of fluorine to some samples as sodium fluoride, then ashing and testing for fluorine. The calculated amount of fluorine was recovered in each case. From this it was reasoned that there was nothing present interfering with the determination and that being the case there was no fluorine present.

*Diet formulae.*—With the above salts and organic materials the experimental diets adequate in all the factors known to be required for growth and reproduction were made up. The composition of the diets is shown in Table III.

<sup>2</sup> Northwestern Yeast Company's dried yeast.

<sup>3</sup> Mead's Viosterol 250D.

## CARE OF THE RATS

The rats used in this investigation were the McCollum strain which has been inbred for over eighteen years. The rats were kept in a special room where no roach powder was used. Sixteen rats from three different litters, about thirty-five days of age, and weighing about 50 gm. each, were chosen for the experiment. Ten rats, five males and five females, were fed the low fluorine ration, while six, three males and three females, were fed the same ration with 0.001 per cent fluorine added. The rats had access to the ration at all times. Distilled water also was kept before them at all times. Once a week iodine (4.2 mg. per 100 cc.) was added to the drinking water to insure an ample supply of that element. The rats were all weighed once a week. Females were placed in individual cages when they became pregnant and were given filter paper clippings for nest material (this was found to be free from fluorine).

TABLE IV  
REPRODUCTION RECORD (TOTAL LITTERS)

Series	Second Generation			Third Generation		
	Number of litters	Number of rats	Number rats lived	Number of litters	Number of rats	Number rats lived
Low F	8	64	24	9	63	5
Control	5	46	7	5	30	0

## CALCIUM AND PHOSPHORUS DETERMINATIONS

Calcium determinations were made by McCrudden's (13) method, which consists of the precipitation of calcium oxalate and titration with potassium permanganate. Phosphorus was determined by the volumetric method of the A. O. A. C. (14). Determinations were made upon a solution of the ash of the bones. From 0.3 to 0.5 gm. of ash were dissolved in dilute nitric acid and the solution made up to a volume of 200 cc. Aliquots were taken of this stock solution for both the calcium and phosphorus determinations.

Calcium and phosphorus determinations were made on the bones only. All the teeth were used for fluorine determinations or for histological study. Determinations were made on the combined ashes of tibiae, fibulae, femurs and humeri. Calcium determinations were made in triplicate and phosphorus determinations in duplicate.

TABLE V  
REPRODUCTION RECORD (FIRST LITTERS)

Series	Second Generation			Third Generation		
	Number of litters	Number of rats	Number rats lived	Number of litters	Number of rats	Number rats lived
Low F	5	43	12	8	58	0
Control	3	27	0	5	30	0

TABLE VI  
FLUORINE AND ASH CONTENT OF TEETH AND BONES

Description	Teeth		Bones	
	% Ash	% F in Ash	% Ash	% F in Ash
Stock animals				
2 yr. old females			60.9	0.051
1 yr. old females	68.20	0.018	60.1	0.061
12-18 mo. old	69.9	0.021	61.4	0.075
Young males	66.5	0.0025	58.5	0.070
35 days old	65.7	0.006	49.2	0.02
45 days old	64.80	—	47.5	0.035
28 days old	61.6	0.000?	45.9	0.00?
Not stock, but normal 50 days old (on an experimental diet not analyzed)			50.69	0.01
Low F Animals				
(4 mos.)	<div> <div>Lower</div> <div>73.7</div> <div>Upper</div> <div>71.5</div> </div>	<div> <div>&lt;0.0005</div> <div>&lt;0.0005</div> </div>	64.9	0.015
Diet No. 2				
Male, 3½ mos.	75.33*	<0.0005	60.02	0.0046
Female, 4½ mos.			60.09	0.0046
Male, 4½ mos.			61.86	0.0027
2nd. gen. 8 wks.			58.74	0.001
2nd gen. 3 mos.			63.3	0.0045
Diet containing .001% F added				
Female, 3½ mos.			65.72	0.120
Male, 3½ mos.			65.11	0.056

\* Combined to give a larger sample.

## OBSERVATIONS ON THE EXPERIMENTAL ANIMALS

*Gross Observations.*—The rats on the low fluorine diet looked very well, were quite fat and on the whole appeared normal. No difference could be noted between these and the control rats which were fed fluorine. The hair of the low fluorine rats was perhaps a little coarser than that of the control rats but not enough to be striking. The incisors of both series of animals were of a deep orange color on the ventral surface. The superior incisors were darker than the inferior. The teeth showed no caries and from gross appearances seemed to be perfect.

TABLE VII  
CALCIUM AND PHOSPHORUS CONTENT OF RAT BONES

Substance	% Ash	% F	% Ca	% P	Ca/P
Bones, female, F fed	65.72	0.12	37.21	17.81	2.09
Bones, male, F fed	65.11	0.056	36.97	17.70	2.09
Bones, male, low F	61.86	0.0027	38.61	18.23	2.12
Bones, female, low F	60.09	0.0046	37.79	18.24	2.07
Bones, males, low F	60.37	0.003	36.88	18.42	2.00
Bones, males and females, low F	58.74	0.001	36.67	18.42	1.99
Bones, normal young	50.69	0.01	35.64	19.45	1.83
Bones, 2 low F	60.02	0.0046	37.43	18.11	2.07
Bones, normal old females	60.12	0.061	36.92	17.20	2.15
Bones, 1 yr. old females	60.85	0.051	36.97	17.20	2.15
Bones, low F, females	63.30	0.0045	37.63	18.07	2.08
Bones, normal young	47.48	0.035	35.40	18.74	1.89

Reproduction was quite normal. The litters varied in size from five to ten young; the young seemed normal when born and weighed from four to five grams each. The mother in almost every case accepted the young and made a nest in the filter paper clippings provided. However, in many instances the first litter and in some instances the second litter died in from one to two days. Presumably it was starvation since no milk could be seen in the young. However, in some cases the young would obtain milk, live for three or four days and then die. The control animals behaved in the same way. Table IV shows the number of litters, the numbers of rats born and the number that lived in the second and third generations. Table V shows the number of first litters, the number of rats born and the number of rats that lived in the second and third generations.

Many of the rats on the low fluorine diet lost their tails when quite young. The tail would become constricted and the end would either dry up and drop off or would become oedematous and finally break off at the

constriction. This phenomenon did not appear in more than about half the rats on the low fluorine diet and was not observed at all in the controls. The phenomenon was seen only in rats one to three weeks of age. It has been occasionally observed in the stock colony of the laboratory and can hardly be related to fluorine deficiency.

*Fluorine Determinations.*—Fluorine determinations were made on the teeth and bones of stock rats, of rats on low fluorine diet and of rats on the low fluorine diet with 0.001 per cent fluorine added. Fluorine determinations were also made on stock rats of different ages in order to find out whether or not fluorine accumulates in the bones and teeth. Determinations were made on experimental rats which corresponded as nearly as possible in age with the stock rats used. Determinations were also made on both the first and second generations of the experimental rats. The results are tabulated in the following table (Table VI) which also shows the per cent of ash. All fluorine determinations are reported as per cent of the ash.

*Calcium and Phosphorus Determinations.*—Calcium and phosphorus determinations were made on the ash of the bones of stock rats of different ages and of the experimental rats. All calcium determinations were made in triplicate and phosphorus determinations in duplicate. The results are shown in Table VII, together with the per cent fluorine and the calcium: phosphorus ratio. Calcium and phosphorus determinations were not made on teeth because of lack of material.

The gross appearance of the teeth of the fluorine-free animals showed no deviation from the normal.

#### DISCUSSION OF RESULTS

The diet was very low in fluorine but not quite free, as shown by the analyses of the bones and teeth.

Much trouble was experienced in getting the young to live. Moreover, fluorine could not be the cause of death since the control rats receiving fluorine behaved in the same manner as did the rats on the fluorine-free ration. In fact the results shown in Tables IV and V even favor the absence of fluorine. It would seem that there was some factor lacking or some factor present that produced a condition which was not favorable for the survival of the young.

Table VI shows that there is normally present a relatively large amount of fluorine in the bones and teeth of the rat, there being more in the bones than in the teeth.

The diet plays a large part in determining the fluorine content of the

bones. The bones of rats on an experimental diet, not analyzed but adequate in every respect, gave a value of only 0.01 per cent fluorine while those on the stock diet gave values of nearly 0.04 per cent for a corresponding age. Bones of rats on the experimental low fluorine diet gave values of 0.001 to 0.0046 per cent or approximately 6 to 25 parts per million of the dry bone. The fluorine content of the bones of rats on the stock diet seemingly does not rise above 0.08 per cent. It will also be seen that as small a quantity as one milligram of fluorine per hundred grams of food added to the low fluorine diet causes as much or more fluorine to be deposited in the bones as does the stock diet.

The values found for the fluorine content of the teeth are extremely interesting in view of the fact that it is in the teeth that the first and most important effects are noticed when an excess of fluorine is fed in the diet. It will be seen from Table VI that there is normally in the stock rats somewhat less fluorine in the teeth than in the bones. Furthermore, no fluorine could be detected in the teeth of the animals on the low fluorine diet. Thus, if fluorine is present as a factor in the consolidation of the tooth, abnormalities should appear in the histological picture. There is, however, no marked abnormality in the structure. The teeth seem to be excellent, no indication of caries, and seemingly perfect calcification. There is some slight indication of hemorrhage and a proliferation of capillaries in the bone just outside of the outer enamel epithelium. If such is the case, there should be some indication of abnormalities in the long bones and in the marrow. Work is being continued along that line.

The calcium and phosphorus determinations shown in Table VII do not show anything striking. The calcium to phosphorus ratio which has been shown to change slightly with the feeding of an excessive amount of fluorine cannot be said to have changed at all. The ratio is slightly higher than that reported by McClure and Mitchell, but it is practically the same for the rats on the stock diet, on the low fluorine diet and on the diet containing added fluorine.

#### CONCLUSIONS

1. A modified method for the colorimetric determination of fluorine is presented.
2. Young rats (16–18 days) contain little if any fluorine.
3. Rats normally contain considerable fluorine in their bones and teeth.
4. Within certain limits the fluorine content of bones and teeth increases with age.
5. The fluorine content of teeth and bones varies with the diet fed.



6. Rats grow normally on a diet low in fluorine.
7. A diet low in fluorine does not affect reproduction in any way.
8. The fluorine content of bones can be reduced to between six and twenty-five parts per million and can be eliminated from the teeth, insofar as can be determined, without showing any gross deleterious effect.
9. There is no striking structural change in teeth free from fluorine from rats on a low fluorine diet.
10. There is some slight indication of a proliferation of capillaries in the tooth pulp and surrounding bone. A similar condition has been at times noted even in the stock animals.
11. There is no change produced in the calcium to phosphorus ratio in the bones by feeding a diet very low in fluorine.

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## STUDIES ON GROWTH

### I. GROWTH FACTORS IN LIVER\*

By

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THE known facts regarding the nutritive requirements of man and of the lower animals have increased remarkably within the past decade. However, this newer knowledge has not yet made possible a complete understanding of normal nutrition so that at the present time one is still unable to state definitely the nature of an adequate food mixture. The experimental data of investigations in which the white rat has been used have been made particularly confusing by the changes which have been observed recently in the rate of growth of stock or control animals (1, 2, 3). Obviously, results become increasingly difficult to interpret as the standard growth curves or norms vary. An additional complicating factor in some cases has been the acceptance of a fair degree of growth of rats on an experimental diet as satisfactory even though the rate of growth was considerably below that of stock or control rats. The assumption has been made in such cases that artificial mixtures of purified food materials must of necessity be inferior to a mixture of natural foods. It appeared desirable to test the validity of this assumption and, therefore, an attempt has been made to find a satisfactory combination of supplements which would permit optimum growth on a simple basal diet. Inasmuch as liver has been shown by Osborne and Mendel (4, 5) to exert a pronounced stimulating effect upon growth, the experiments have centered around the use of this material as a dietary supplement and an investigation is being made of its water-soluble growth factors.

Twenty different combinations of supplements were tested and all but one permitted a rate of early growth as good or better than that previously recognized as normal (6). It may be stated that the capacity of a supple-

\* Preliminary reports of this work were presented before the American Society of Biological Chemists at Montreal, April, 1931 (Graham, C. E. and Griffith, W. H., *Jour. Biol. Chem.*, 1931, 92, 63, and before the Society for Experimental Biology and Medicine, St. Louis (Graham, C. E. and Griffith, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1929, 26, 715; 1929, 26, 862; 1931, 28, 756; 1931, 28, 1086).

ment to stimulate appetite was found to be the principal cause of differences between the various groups. This is in agreement with the conclusion of Palmer and Kennedy who, although unsuccessful in devising a synthetic diet equivalent to a natural diet (7, 8), showed that the difference between the two diets depended upon consumption of food and not upon utilization of food (9). The experiments reported in this paper not only demonstrated the possibility of obtaining optimum growth on a simplified diet but they also emphasized the importance of certain dietary essentials in accelerating the rate of growth through a stimulation of appetite.

#### EXPERIMENTAL PROCEDURES

The simplified food mixture introduced by Evans and Burr (purified casein 25; commercial sucrose 75; salt mixture 4) (10) was chosen as the basal diet. The Osborne and Mendel salt mixture (11) was used and was modified by the addition of 0.2 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per kilo. of salt mixture. Norwegian cod liver oil was employed as a source of vitamins A and D. In a few experiments wheat germ oil, prepared by the ether extraction of wheat embryo, was used. The water-soluble vitamins were furnished by one or more of the following: dried starch-free baker's yeast; dried hog liver; dried autoclaved hog liver; tikitiki, an extract of "rice polish" prepared according to Wells (12); yeast extract, an extract containing the antineuritic factor prepared according to Seidell (13); and, an aqueous extract of hog liver.<sup>1</sup> The autoclaved hog liver was prepared by heating fresh hashed liver at 120°C. and 15 lb. for five hours. At the end of this operation the pH ranged from 4 to 5. This material, as well as the whole liver and whole yeast, were dried in a warm air dryer at 50°C.

Young male rats were used, averaging 28 to 30 days of age and 49 to 51 gm. in weight. The rats were kept in individual raised cages except when stated otherwise. Twenty different combinations of supplements were investigated (Table I), each variation being tested on a group of ten or more rats. With one exception no changes were made in the supplements furnished a group during the course of an experiment. The experimental periods ranged from 60 to 200 days. The dry basal diet was fed *ad libitum* in small dishes resembling the McCollum feeding cup and wasting of food was not encountered. The supplements were fed daily in Syracuse dishes.

The consumption of water was measured as well as the gain in weight and the consumption of food. Water was supplied from inverted bottles having delivery tubes with an inside diameter of 5 mm.

<sup>1</sup> We wish to thank Mr. P. A. Katzman for the preparation of the liver extract.

TABLE I  
COMPOSITION OF SUPPLEMENTS SUPPLIED THE GROUPS OF MALE RATS

Group	No. of rats	Average starting		Cod liver oil (gm.)	Yeast (gm.)	Additional daily supplements
		Age (days)	Weight (gm.)			
1	10	29	51	0.09	0.50	—
2	14	29	53	0.27	0.50	—
3	10	30	51	0.36	0.50	—
4	10	30	50	0.09	0.50	0.18 gm. of lard
5	9	27	51	0.27	0.50	0.09 gm. of lard
6	10	29	53	0.27	0.50	0.09 gm. of wheat germ oil
7	10	30	50	0.27	0.50	10% of lard in basal diet
8	14	31	52	0.27	0.50	(Cages on shavings)
9	10	31	51	0.27	0.50	(Commercial casein in basal diet)
10	10	31	55	0.27	0.50	0.25 cc. of tikitiki
11	19	27	52	0.27	1.00	—
12	10	31	51	0.27	1.00	0.5 cc. of liver extract
13	10	30	50	0.27	0.50	0.5 cc. of liver extract+0.09 gm. of wheat germ oil.
14	10	29	55	0.27	0.50	0.5 gm. of whole dried liver
15	10	30	49	0.27	0.15	0.85 gm. of whole dried liver
16	10	28	49	0.27	0.00	1.0 gm. of whole dried liver
17	12	27	52	0.27	0.00	0.21 gm. of autoclaved liver+0.25 cc. of tikitiki
18	12	28	52	0.27	0.00	1.0 gm. of autoclaved liver+0.25 cc. of tikitiki
19	10	29	52	0.27	0.00	1.0 gm. of autoclaved liver+0.5 cc. of yeast extract.
20	10	29	51	0.27	0.50	1.0 gm. of autoclaved liver
21	10	29	54	0.00	0.00	(Stock diet)

The caloric value of the total ingested food was calculated on the basis that the basal diet, yeast and liver furnished 3.75, 2.5 and 3.75 calories per gram respectively. Supplements of cod liver oil, lard and wheat germ oil were included on the basis of 9 calories per gram. Supplements of tikitiki, liver extract and yeast extract were not included in the calculation of the caloric intake.

The coefficients of digestibility of the stock diet and of the adequately supplemented basal diet were determined according to the method of Armsby (14) in which the ratio of dry feces to dry food is found. The results showed the stock and basal diets to be 76.5 and 98 per cent digestible respectively. On the basis of these results the necessary correction was made for the caloric intake of the rats on the stock diet and the experi-

mental diet was assumed to be wholly digestible. The stock diet used in our colony is a commercial dog biscuit which has been found adequate for growth, reproduction and lactation.

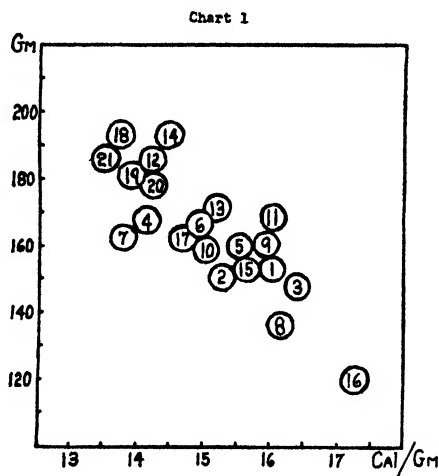


CHART 1. The average gain in weight during the first 60 days (30 to 90 days of age) of groups listed in Table I plotted against calories required per gram gain in weight.

The extensive data obtained in these experiments are summarized in Tables I to V and Charts 1 to 3. The results are presented as group averages since it was obviously impracticable to give the complete data for each

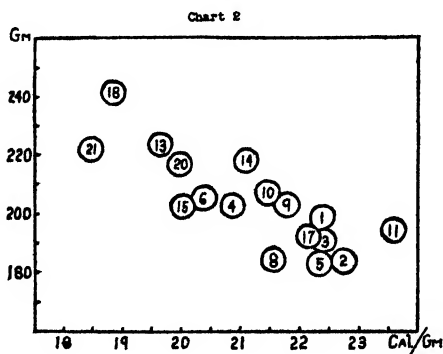


CHART 2. The average gain in weight during the first 100 days (30 to 130 days of age) of groups listed in Table I plotted against calories required per gram gain in weight.

of the 270 rats. It was recognized that larger groups would have been more desirable in view of the individual variations which appear even among litter mates. However, it was believed that the conclusions which were

drawn from the data were significant since they were not dependent upon the results of any one group.

Although a record of the gain in weight and of the food and water intake was made for each rat for every 10 or 20 day interval, the results were combined in the tables into three periods, the first 60 days, the first 100 days and the period exceeding 100 days. The 60 day period represented the period of most rapid growth while the 100 day period represented the period of rapid growth and the attainment of nearly adult size. For these

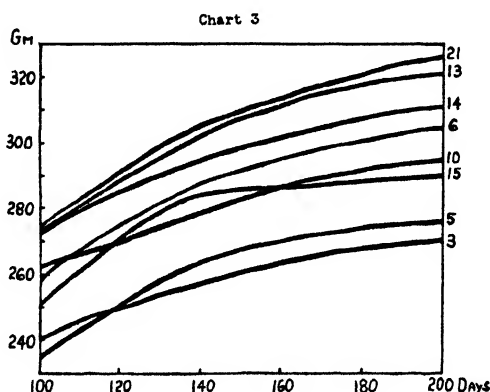


CHART 3. The average growth curves during the second 100 day period (130 to 230 days of age) of some of the groups listed in Table I. The number of the group is indicated on each curve

two periods the efficiency of utilization of food was expressed in terms of calories required to increase one gram in weight. The period exceeding 100 days represented an interval of slow growth in which a much higher proportion of the ingested food must have been used for maintenance than was the case in the periods of rapid growth. For the later period the ratio of calories to gain in weight has little significance. In order to compare the utilization of food under the influence of the various supplements during this third period, two "efficiency quotients" were calculated. E.Q.I represented the calories consumed per day per 100 gm. of body weight and was calculated as follows:

$$\text{E.Q.I} = \frac{\text{Total calories} \times 100}{\text{Mean weight} \times \text{Length of period in days.}}$$

E.Q.II represented the calories consumed per day per gram of gain in weight per 100 gm. of body weight and was calculated as follows:

$$\text{E.Q.II} = \frac{\text{Total calories} \times 100}{\text{Gain in weight} \times \text{Mean weight} \times \text{Length of period in days.}}$$

E.Q.II was patterned after that suggested by Palmer and Kennedy (9) and differs only in the use of total calories per day in place of total grams of dry food.

It was assumed in the consideration of the results that vitamins B and G were the water-soluble vitamins required by the rat since only these two have been definitely established as members of the B complex (15, 16). The autoclaved liver was found to be a rich source of vitamin G and was probably free from vitamin B.<sup>2</sup> The tikitiki, as was shown by Evans and Burr (17, 18), was an excellent source of B which contained not more than a trace, if any, of G.<sup>2</sup>

Although many interesting possibilities were suggested by the results which have been obtained, it appeared desirable to point out only certain of the more important relationships. These will be discussed under the following headings:

1. The effect of liver on the rate of growth and on the consumption and utilization of food.
2. The consumption of water.
3. The occurrence of feet and tail signs.

*1. The effect of liver on the rate of growth and on the consumption and utilization of food*

Whole dried liver (1.0 gm. daily) was not an adequate source of the water-soluble vitamins although this supplement permitted some growth (Table II, Group 16). Substitution of 0.15 gm. of the liver with an equal quantity of yeast (Group 15) improved growth but not so much as substitution of half of the liver with yeast (Group 14). It was evident that the limiting factor in liver was B since excellent growth resulted if the supplement contained autoclaved liver and a source of B such as tikitiki (Group 17 and 18) or yeast extract (Group 19). Group 18 grew as well as the control group (Group 21) which indicated that 1.0 gm. of autoclaved liver was adequate as a source of G during the first 100 days on the experimental diet.

The effect of liver in accelerating the rate of growth is clearly shown in the last column of Table II which gives the number of days required to attain a weight of 200 gm. from a starting weight of approximately 50 gm. The only groups which required less than 50 days were those receiving liver in some form (Groups 12, 13, 14, 18, 19 and 20). The only groups which received liver and required more than 50 days were the two groups

<sup>2</sup> Unpublished experiments.

mentioned above as being definitely low in the supplement of B (Groups 15 and 16) and Group 17, which received only 0.21 gm. of autoclaved liver daily. The significance of this effect of liver becomes more important upon examination of the results with Groups 1 to 11, none of which received liver and all of which required more than 50 days to reach 200 gm.

TABLE II  
AVERAGE WEIGHT OF GROUPS OF MALE RATS AND AVERAGE FOOD AND WATER CONSUMPTION  
DURING 60 AND 100 DAY PERIODS

Group	Results for first 60 days				Results for first 100 days				Days to reach 200 gm.
	Final Weight		Total food (cal.)	Total water (cc.)	Final Weight		Total food (cal.)	Total water (cc.)	
	Range (gm.)	Average (gm.)			Range (gm.)	Average (gm.)			
1	174-233	203	2405	1093	225-291	249	4438	2116	60
2	188-240	204	2355	1081	206-272	236	4155	2158	62
3	137-245	198	2403	1231	204-304	241	4238	2346	62
4	178-260	217	2375	944	219-305	253	4245	1722	52
5	189-216	203	2397	910	219-265	235	4132	1786	61
6	196-238	217	2452	895	220-290	258	4197	1775	51
7	172-245	212	2235	1033	—	—	—	—	—
8	162-208	187	2195	1268	200-258	236	3980	2424	70
9	175-256	210	2525	1227	208-310	254	4443	2389	57
10	158-258	214	2385	1232	224-326	262	4455	2484	55
11	178-259	218	2670	1292	205-278	247	4600	2368	55
12	209-270	235	2620	1459	—	—	—	—	45
13	194-255	219	2557	1113	249-315	273	4382	2080	48
14	197-287	247	2790	1159	218-316	274	4635	2084	41
15	157-225	204	2415	1045	216-285	252	4105	2009	61
16	148-188	168	2064	914	—	—	—	—	—
17	183-256	215	2403	1136	210-273	245	4309	1930	68
18	199-289	243	2607	1279	249-347	294	4547	2525	44
19	184-272	233	2542	1216	—	—	—	—	46
20	192-282	230	2527	1265	220-331	268	4342	2373	46
21	198-278	241	2540*	1423	224-300	275	4052*	2513	38

\* 76.5 per cent of actual caloric intake.

The superiority of the liver supplement is also shown in Charts 1 and 2 in which the 60 and 100 day weight increments are plotted against the calories required to gain one gram in weight. Groups 1 to 11 received a minimum of 0.5 gm. of whole dried yeast as a source of vitamins B and G. This was inadequate for optimum growth. Furthermore, an increase or decrease in cod liver oil (Groups 1, 2 and 3), the addition of lard (Groups 4, 5 and 7), the addition of wheat germ oil (Group 6), access to feces



(Group 8), the use of commercial casein (Group 9), the addition of tikitiki (Group 10) and the doubling of the amount of yeast (Group 11) were all ineffective in bringing about optimum growth.

The optimum early growth due to the presence of liver in the daily supplement was associated with an increased appetite and with an apparently more efficient utilization of food. The data for food consumption for the first 60 days show that nine of the eleven groups which did not receive liver (Groups 1 to 11) consumed less than 2500 calories whereas six of the

TABLE III

AVERAGE WEIGHT OF GROUPS OF MALE RATS AND AVERAGE FOOD AND WATER CONSUMPTION DURING EXPERIMENTAL PERIOD AFTER FIRST 100 DAYS

Group	Experimental period (days)	Mean weight (gm.)	Final weight (gm.)	Gain per day (gm.)	Calories per day	Water per day (cc.)	E.Q.* I	E.Q.** II
3	100-200	256	270	0.29	45.8	25.5	17.9	0.62
4	100-180	260	267	0.18	41.6	19.8	16.0	0.91
5	100-200	256	276	0.41	44.8	21.5	17.5	0.43
6	100-200	282	305	0.47	44.2	21.8	15.7	0.33
10	100-200	279	295	0.33	47.9	27.0	17.1	0.52
11	100-160	268	278	0.33	47.8	30.7	17.8	0.54
13	100-200	297	321	0.48	48.6	23.3	16.4	0.34
14	100-200	292	310	0.36	45.4	24.1	15.5	0.43
15	100-200	271	290	0.38	44.4	24.2	16.3	0.43
17	100-160	252	259	0.23	38.7	24.5	15.3	1.10
18	100-180	302	310	0.19	48.8	22.4	16.1	0.85
20	100-180	276	284	0.20	45.1	26.7	15.9	0.80
21	100-200	301	326	0.51	42.2	29.9	14.0	0.27

$$* \text{ E.Q. I} = \frac{\text{Calories per day} \times 100}{\text{Mean weight}}$$

$$** \text{ E.Q. II} = \frac{\text{Calories per day} \times 100}{\text{Gain} \times \text{Mean weight}}$$

nine groups receiving liver consumed more than 2500 calories. The three groups receiving liver and consuming less than 2500 calories were, again, the groups furnished supplements which were definitely inadequate (Groups 15, 16 and 17). Inspection of Chart I shows that eight of the eleven groups not receiving liver (Groups 1 to 11) required 15 or more calories per gram of gain in weight while six of nine groups receiving liver required less than 15 calories per gram of gain. It is realized that these figures are not necessarily indicative of variations in the efficiency of utilization of food, because of the number of variables concerned. However,

the data do relate rapid early growth and increased food consumption to the presence of liver in the daily supplement.

The effect of liver was not only evident in accelerating the rate of early growth but was also related to the continuation of growth after the first 100 days. Of the four groups which averaged more than 300 gm. at the termination of the experimental period, three consumed diets containing liver in some form (Table III and Chart 3). Table III shows the average daily food consumption and two efficiency quotients for experimental periods in excess of 100 days. Such quotients may have real value if comparisons are made on rats of the same weight or on rats receiving the same number of calories. The actual significance of the quotients given in Table III cannot be determined until more definite information is available concerning the relative caloric requirements of maintenance and growth. It was of interest to find that of the seven groups which were continued on the experimental diets for 200 days, the average daily caloric intakes were 41.5, 43.1 and 45.9 calories per day for the 60 day, the 100 day and the 100-200 day periods respectively.

TABLE IV  
RATIO OF TOTAL WATER CONSUMPTION TO TOTAL FOOD CONSUMPTION  $\left( \frac{\text{cc.}}{\text{calories}} \right)$

Group	0 to 60 days	0 to 100 days	Period over 100 days
1	0.45	0.48	—
2	0.46	0.52	—
3	0.51	0.55	0.56
4	0.40	0.41	0.48
5	0.38	0.43	0.48
6	0.37	0.42	0.49
7	0.46	—	—
8	0.58	0.61	—
9	0.49	0.54	—
10	0.52	0.56	0.56
11	0.48	0.52	0.64
12	0.56	—	—
13	0.44	0.48	0.48
14	0.42	0.45	0.53
15	0.43	0.49	0.54
16	0.44	—	—
17	0.47	0.45	0.63
18	0.49	0.56	0.46
19	0.50	—	—
20	0.50	0.55	0.59
21	0.56	0.62	0.71

A specific demonstration of the effect of liver on appetite is seen in the comparison of Groups 17 and 18 which received the same amount of tiki-tiki but different quantities of autoclaved liver. Group 18 consumed more food in all three periods.

## 2. The consumption of water

The total average consumption of water during the first two periods is given in Table II and the average daily consumption of water in the third period is given in Table III. Table IV shows the water intake per calorie of food consumed for the three periods. It is quite apparent from this table that a close relationship existed between the amounts of food and water consumed. The value of the ratio increased slightly with the age of the rats, the average values for the 7 groups on the experimental diets for 200 days being 0.44, 0.48 and 0.51 cc. per calorie for the three periods.

TABLE V  
NUMBER OF RATS SHOWING FEET OR TAIL SIGNS

Group	20 days			60 days			100 days			200 days		
	+	±	-	+	±	-	+	±	-	+	±	-
1	3	4	3	5	5	0	9	1	0	0	1	9*
2	8	1	5	11	3	0	12	1	1	—	—	—
3	5	4	1	9	0	1	10	0	0	1	0	8
4	0	2	8	1	3	6	0	0	10	0	0	10
5	3	1	5	3	4	2	4	2	3	0	2	7
6	2	5	3	0	4	6	0	1	9	0	0	10
7	0	0	10	0	2	8	—	—	—	—	—	—
8	0	0	10	2	7	5	5	7	2	—	—	—
9	0	1	9	7	2	1	9	0	1	—	—	—
10	4	2	4	10	0	0	10	0	0	1	3	6
11	4	4	2	7	3	0	9	1	0	3	4	3**
12	3	5	2	10	0	0	—	—	—	—	—	—
13	1	0	9	0	3	7	0	2	8	0	0	10
14	0	4	6	0	4	6	0	3	7	0	1	9
15	0	2	8	0	4	6	0	4	6	0	0	10
16	0	0	10	0	2	8	0	1	9	—	—	—
17	0	1	11	2	9	1	0	4	8	0	0	12**
18	2	5	4	0	8	4	0	3	9	0	0	12†
19	2	3	5	2	5	3	—	—	—	—	—	—
20	0	6	4	0	8	2	0	4	6	0	0	10†
21	0	0	10	0	0	10	0	0	10	0	0	10

\* Rats discontinued at 140 days.

\*\* Rats discontinued at 160 days.

† Rats discontinued at 180 days.

### 3. *The occurrence of feet and tail signs*

Dermatitis of the feet and tail was of common occurrence among the experimental rats (Table V). The "feet and tail signs" consisted of dry scaliness of the feet, of the hind feet particularly, and scaliness and soreness of the tail. The signs were apparently identical with those described by Burr and Burr (19). In some rats the feet signs appeared first, in others the tail signs preceded the feet signs. In most instances the signs appeared within 20 days, were most severe between 40 and 60 days and slowly disappeared so that they were scarcely evident at 200 days except in those rats which had lost sections of the tail.

The signs were very severe in the rats receiving cod liver oil and 0.5 gm. of yeast daily (Groups 2 and 3) and were not prevented by doubling the yeast (Group 11), by using commercial casein (Group 9), by adding tikitiki (Group 10) or by adding liver extract (Group 12). The signs were absent on the stock diet (Group 21). Wheat germ oil, whole liver, and lard were effective in decreasing the severity of the signs (Groups 6, 15 and 7). The failure of the liver extract to prevent the signs (Group 12) indicated that liver fat was possibly the effective agent in whole liver.

Changes in the feet and tail of the rat have been reported by other investigators and appear to be associated with various types of nutritive deficiencies (20 to 25). Although Burr and Burr originally reported that cod liver oil prevented the signs, they later came to the conclusion that the highly unsaturated fatty acids of cod liver oil were used in low fat diets for growth but that the scaly skin was cured only by linolic or linolenic acids which are apparently lacking in cod liver oil (26). Sinclair also noted that the signs were not prevented by cod liver oil but were prevented by lard and also by permitting access to feces (24).

This particular abnormality did not appear to be related necessarily to vitamin B or G deficiency and might therefore complicate the interpretation of results on low fat, low G diets. In this connection the possible presence of toxic substances in experimental diets may be important. Such injurious compounds have been suggested by the work of Norris and Church (27), Parsons (22) and Fixsen (28).

### DISCUSSION

These results have demonstrated that, for this colony of rats at least, the growth between the ages of 30 and 230 days on the highly purified Evans and Burr diet can equal that on a diet of mixed natural foods. The special supplements which permitted such optimum growth and

which, therefore, supplied the required appetite and utilization factors were cod liver oil and some source of vitamin B, such as yeast, yeast extract or tikitiki, and liver as a source of vitamin G. Optimum growth, which was associated with increased food consumption, appeared to depend upon the presence of the liver. The growth stimulating properties of liver have been observed by several investigators (29 to 32) since the earlier reports of Osborne and Mendel (4, 5).

Although these experiments were not originally intended to determine the role of fat in experimental diets, the results suggested that lard and particularly wheat germ oil exerted beneficial effects not shown by cod liver oil. The daily addition of 3 drops of wheat germ oil to the usual supplement of cod liver oil (Group 6) markedly improved the rate of growth, especially during the third period. Group 6 was the only group not receiving liver to exceed 300 gm. in weight. This group showed only fair growth during the first 60 days, better growth during the first 100 days and nearly optimum growth during the period in excess of 100 days. Group 13 which received wheat germ oil and liver extract showed growth nearest the optimum during the third period. Whether this effect on growth was due to vitamin E (33) is not known. However, the fact that 9 of 10 female rats on the experimental diet supplemented with yeast and cod liver oil produced litters not only once but also after a second mating (unpublished data) was considered as evidence of the presence of vitamin E in the diet, presumably in the cod liver oil (34). In view of the observation of Burr and Burr (19) on the effect of fat free diets on the water intake of rats it was interesting to note the apparently decreased water intake of the groups receiving lard and wheat germ oil (Table IV, Groups 4, 5 and 6). In general, the results were in agreement with those of McAmis, Anderson and Mendel (23) who concluded that small amounts of fat were beneficial in some unrecognized manner in low fat diets. They were also in accord with the findings of Burr and Burr (26) that fatty acids other than those in cod liver oil were required by the rat. It is possible that the effectiveness of the whole liver was due in part to the liver fat.

As has been stated, 0.5 gm. of whole yeast daily supplied water-soluble factors sufficient for fair growth. The failure of 1.0 gm. of yeast materially to better the growth obtained with 0.5 gm. was difficult to understand. Evans and his co-workers (35, 36) also observed that 1.0 gm. was not superior to 0.7 gm. of yeast although the addition of liver to the smaller quantity of yeast resulted in improved growth. These results with yeast emphasized the advantages of liver over yeast as a source of vitamin G.

Autoclaved yeast or autoclaved marmite have been the usual sources of G in experimental work ever since Goldberger demonstrated the effectiveness of this material in preventing pellagra. As was shown by Quinn (37) the relative amounts of B and G in different yeasts may show wide variations. Moreover, Williams, Waterman, and Gurin (38) have demonstrated that the vigorous treatment, particularly the alkaline autoclaving, required to destroy all of the B of yeast also destroyed a large part of the G. Liver, on the other hand, contains only a small amount of B and does not require autoclaving at pH 9 to give a product free from B. Undoubtedly, autoclaving of fresh liver does destroy some of the G but the resulting material has been found to be an excellent source of G. Guha (33) has also recognized the advantages of liver over yeast as a source of G.

Yeast has been widely used in nutrition investigations as a supplement which supplied the vitamin B complex and also as a source of material for experiments in which separation of active fractions has been attempted. The failure of some yeasts, at least, to permit more than fair growth demands serious consideration. Inasmuch as erroneous conclusions may be drawn from experiments in which comparisons are made with "standard or normal" growth curves representing only fair growth and not the actual growth capacity of the rats, it would appear imperative to demonstrate that optimum growth is possible on any chosen artificial diet before the diet is subjected to further examination.

There was no good evidence in these experiments for the existence of members of the B complex other than B and G. It was realized that the failure with yeast suggested an additional appetite factor in liver which was absent from yeast. However, 0.5 gm. of yeast did supply appetite and utilization factors sufficient for fair growth. The assumption of a new appetite factor in liver would still leave unexplained the failure of 1.0 gm. of yeast to accelerate growth through the increase in the appetite factors manifestly present in 0.5 gm. of yeast. The results suggested the alternative possibility that vitamin G might not be readily available in certain yeasts so that the amount of G actually furnished a rat might have very little relation to the quantity of yeast in the supplement. It is also possible that the larger quantity of yeast introduced some injurious substance which inhibited appetite.

Numerous reports have been made in recent years of the existence of additional members of the B complex (39 to 44) and it is not at all improbable that the occurrence of such factors may be definitely confirmed as a result of future work. Nevertheless, it is believed that certain of these

experiments may be justly criticised for acceptance of very mediocre growth as normal growth and others for failure to demonstrate that adequate amounts of G were actually available in the supplements. Chick and Roscoe (45) have also emphasized the need for caution in interpreting the results of investigations in which autoclaved yeast and particularly alkaline autoclaved yeast were used as a source of G. In this connection it is of interest to find that Hunt (46) has not been uniformly successful in the preparation of residues having the properties previously described and that Williams and Eddy (47) now suggest the possibility that the B<sub>2</sub> of Williams and Waterman (44) might be nothing more than an abundant supply of B.

ADDENDUM.—Since this paper was written several articles have appeared which offer additional support for the occurrence of a new growth factor or of growth factors. As far as we know, there is no evidence which denies the possibility or the probability of the existence of unrecognized members of the vitamin B complex. However, our own results have been of such a nature as to emphasize the need for caution in interpreting observations such as those of Halliday (48) because of the use of autoclaved yeast as a source of G and those of Stiebeling (49) because of the use of experimental animals receiving less than the optimum amount of G. The experiments of Mapson (50) demonstrated anew and in striking fashion the importance of liver as a source of growth factors. Nevertheless, it seems pertinent to raise the question as to whether or not the vitamin G requirement for the consumption and utilization of food for optimum growth has been fully supplied in these experiments.

#### SUMMARY

1. The rate of growth of rats on the highly purified Evans and Burr diet, properly supplemented, equaled that of rats on a diet of mixed natural foods.
2. Optimum growth on the purified diet was directly related to the increased consumption of food resulting from the presence of liver in the daily supplement.
3. Liver was found to be superior to yeast as a source of vitamin G.
4. The ratio of the water intake to the food consumption on the experimental diets was fairly constant and amounted to approximately 0.45 cc. per calorie.
5. Scaliness of the feet and scaliness and soreness of the tail were common on the experimental diet. These signs were less severe if liver or fats

other than cod liver oil were furnished in the daily supplements. This type of dermatitis was not related to vitamin G deficiency.

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## STUDIES ON GROWTH

### II. THE EFFECT OF VITAMINS B AND G ON THE CONSUMPTION AND UTILIZATION OF FOOD\*

By

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**I**N A previous report (2) experiments on growing rats were presented which showed that the amount of the daily supplement of yeast, which was used as a source of the vitamin B complex, influenced the utilization of food. This effect of yeast was observed in experiments in which the food intake was restricted to a stated caloric level and was contrasted with the stimulation of appetite and the resulting acceleration of growth observed if liver was used as a supplement to the diet fed *ad libitum*.

Although the appetite of growing rats is affected by many dietary deficiencies, it has appeared to be most closely related to the vitamin B complex. This relationship was observed by Osborne and Mendel (3) and by Drummond (4) and has been investigated by Karr (5) and by Cowgill (6, 7). Their experiments were performed before the distinction between vitamins B and G had been definitely established. In more recent work Sherman and Sandels (8) found that the loss of appetite in rats was much more pronounced in the case of deprivation of B than of G and concluded that B exerted a specific effect upon appetite whereas the decreased food consumption in G avitaminosis was probably only the "usual concomitant of the weakness, lack of tone, and increasing torpidity of the experimental animals." Burack and Cowgill (9) also concluded that G was not concerned with the stimulation of appetite in dogs. Palmer and Kennedy (10) confirmed the observation of Graham and Griffith (2) that a supplement, such as liver, was mainly effective by a stimulation of appetite and not by an increase in the efficiency of utilization of food. They also demonstrated that there was little difference in the efficiency of utilization of an adequate synthetic diet and of a diet of natural foods.

\* This paper and the previous one of this series (1) are based on data taken from the dissertation submitted by Claire E. Graham to the Graduate School of St. Louis University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

In the first paper of this series (1) it was shown that nearly optimum growth was possible on the properly supplemented Evans and Burr diet and that the optimum growth appeared to depend upon the presence of liver in the daily supplement. Inasmuch as the liver was rich in G and poor in B it was suggested that the effect of liver in increasing the food consumption was related to its content of G and that this vitamin might have some significance in the normal regulation of appetite. In the present series of experiments, in which over 600 rats were used, the effect of yeast and, more particularly, the effect of the vitamin B complex on appetite and the utilization of food have been investigated. The experiments were planned so that it would be possible to compare the appetite stimulants in supplements which resulted in mediocre growth with the appetite factors in supplements which had previously been shown (1) to permit optimum growth.

#### EXPERIMENTAL PROCEDURE

Young male rats averaging 28 to 31 days of age and 49 to 51 gm. in weight were used. The Evans and Burr diet was employed and was supplemented in every case with 0.27 gm. of cod liver oil daily. The experimental period lasted 40 days and most of the groups were restricted to either 975 or 1365 calories during this period. The measured portion of the basal diet and the supplements were fed daily. The sources of the vitamin B complex were whole dried liver, whole dried baker's yeast, dried autoclaved hog liver, and tikitiki.<sup>1</sup> With the exception of one preparation of tikitiki the materials were the same as those used in the experiments reported in the first paper (1).

The charts show the gain in weight during the experimental period, the total calories consumed and the average caloric requirement per gram gain in weight. The latter value represents the efficiency of utilization (for growth) of food and is particularly significant in the comparison of groups having the same caloric intake.

#### EXPERIMENTAL RESULTS

Groups 1 and 2 (Chart 1) were fed tikitiki and autoclaved liver respectively as the sole source of the vitamin B complex. In neither case was there any appreciable growth after the first ten days, although the period of survival was longer in the case of the rats fed the tikitiki. The food intake of those surviving the forty day period was approximately the same

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<sup>1</sup> The same extract of "rice polish" was used in all of these experiments except those listed in Chart 3. The groups in Chart 3 received the tikitiki employed in the earlier experiments (1).

in both groups. It was concluded that the tikitiki and the autoclaved liver were practically free from vitamins G and B respectively.

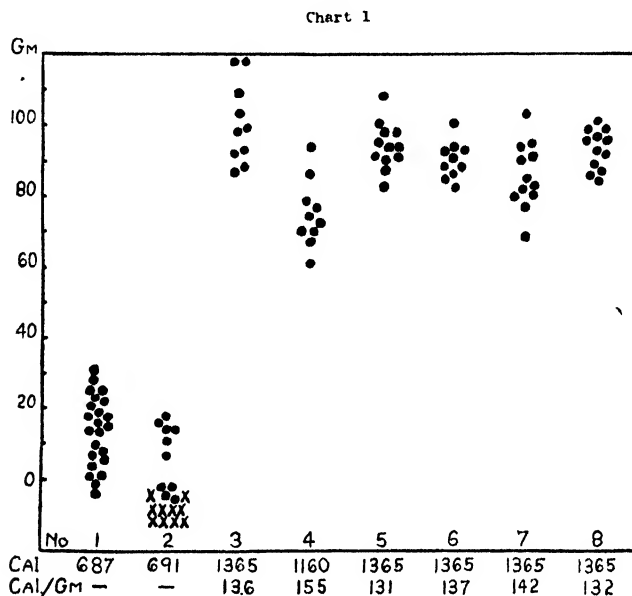


CHART 1. The gain in weight of young rats during a forty day experimental period on the Evans and Burr diet supplemented daily with cod liver oil and the following special supplements:

- No. 1—0.25–0.50 cc. of tikitiki.
- " 2—0.5–1.0 gm. of autoclaved liver.
- " 3—1.0 gm. of whole liver.
- " 4—0.15 gm. of yeast+0.85 gm. of autoclaved liver.
- " 5—0.15 gm. of yeast+0.85 gm. of whole liver.
- " 6—0.2 gm. of whole liver+0.1 cc. tikitiki.
- " 7—1.0 gm. of yeast.
- " 8—0.5 gm. of yeast+0.5 gm. of whole liver.

Groups 1 to 4 were fed *ad libitum* and Groups 5 to 8 were restricted to 1365 calories. Deaths during the experimental period are shown by the letter X.

Groups 3 and 4 were also fed *ad libitum* and were supplied supplements which permitted only mediocre growth. Since it was desired to investigate the utilization of food and the appetite under such conditions, groups were started and restricted to the food intake of Group 3, 1365 calories, during the experimental period. The maximum increase in weight of these rats ranged from 95 to 120 gm. Inasmuch as the same average gain in weight was obtained with a number of quite different supplements (3, 6, and 8, (Chart 1) 11, (Chart 2) 17 and 21 (Chart 3)) it was concluded that this represented the optimum utilization of the 1365 calories. These supple-

ments which permitted the same efficiency of utilization of food had varying effects upon the rate of growth if the basal diet was fed *ad libitum* (1).

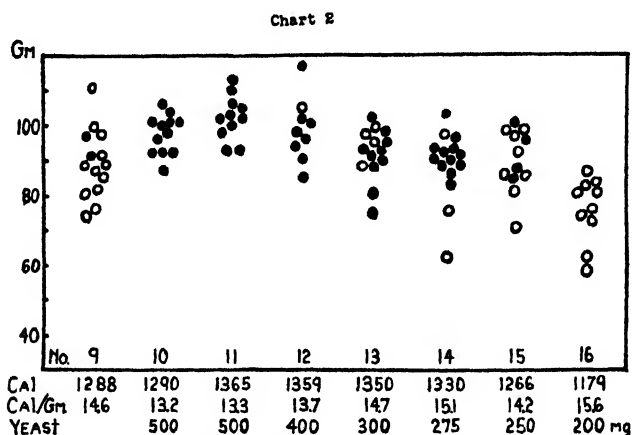


CHART 2. The effect of yeast on the gain in weight and on the consumption of food during the 40 day period. Each group except Group 10 was restricted to 1365 calories. Group 9 received 0.05–0.10 cc. of tikitiki and 0.10–0.15 gm. of autoclaved liver instead of yeast. Circles indicate failure to consume all of the food.

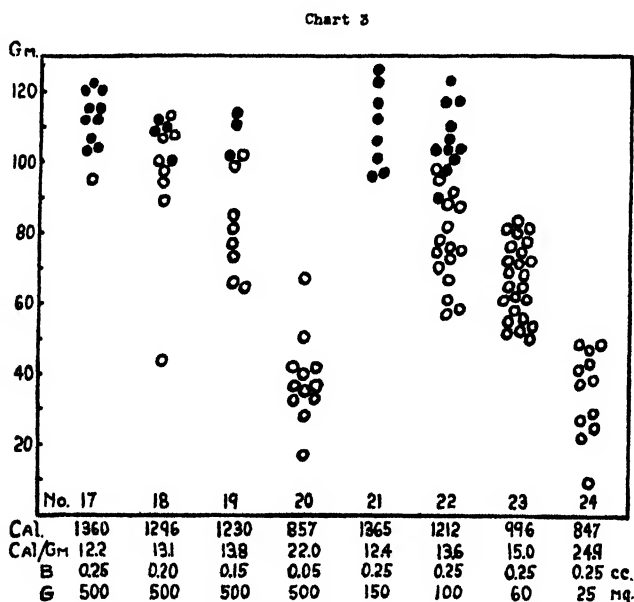


CHART 3. The effect of tikitiki (B) and of autoclaved liver (G) on the gain in weight and on the consumption and utilization of food during the 40 day period. Each group was restricted to 1365 calories. Circles indicate failure to consume all of the food.

Chart 2 shows the effect of yeast as a supplement. Optimum utilization occurred with 0.5 gm. of yeast daily (Group 11). In order to determine whether a small change in the caloric intake would affect the rate of growth, Group 10 was fed 1290 instead of 1365 calories. Comparison of 10 with 11 shows that the smaller caloric intake resulted in a slightly decreased gain in weight. Examination of the results with Groups 11 to 16 demonstrated that the decrease in the quantity of the yeast supplement progressively lowered the efficiency of utilization of food and the gain in weight. Utilization appeared to be affected before appetite since most of the rats in 12, 13 and 14 consumed all of the 1365 calories and yet their average increase in weight was less than that observed if optimum utilization occurred (Group 11).

Chart 3 shows the results obtained in experiments in which the vitamin B complex was furnished by varying amounts of tikitiki (B) and autoclaved liver (G). Groups 17 to 20 received an adequate supply of autoclaved liver and decreasing amounts of tikitiki. Groups 21 to 24 received an adequate supply of tikitiki and decreasing amounts of autoclaved liver. The resulting effect upon appetite was practically identical in the two series since appetite failed if either tikitiki (B) or autoclaved liver (G) was inadequate. These results differed from those in Chart 2 in that failure of appetite was not generally preceded by a marked decrease in the efficiency of utilization of food. A possible explanation of this difference was the fact that the lowering of the yeast supplement (Chart 2) decreased both B and G whereas in the groups in Chart 3 an adequate amount of either B or G was supplied in each case. Group 9 represents an attempt to adjust a daily supplement of tikitiki and autoclaved liver so that the minimum quantities required for the complete consumption of the food (1365 calories) would be supplied. The average food consumption of this group was 1288 calories, practically the same as that of 10 which was restricted to 1290 calories and which received a supplement permitting optimum utilization of food. The poorer utilization of food by 9 indicated that the efficiency of utilization could be decreased by inadequate supplements of autoclaved liver and tikitiki as well as by an inadequate supplement of yeast.

In view of the results which were obtained with a caloric intake of 1365 calories, another series of groups was started in which the caloric intake was restricted to 975 calories during the 40 day period. On this caloric level the maximum gain ranged from 60 to 85 gm. Approximately the same average gain resulted with several different supplements (29, 35 and 39) and it was assumed that this gain represented the optimum utilization of the 975 calories.

Chart 4 shows the results with yeast as the supplement. Optimum utilization occurred with 225 mg., or more, of yeast daily. With less than this amount the efficiency of utilization decreased first and the failure of appe-

Chart 4

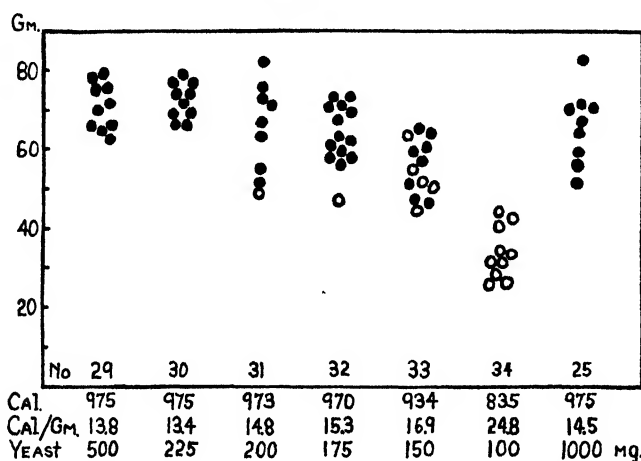


CHART 4. The effect of yeast on the gain in weight and on the consumption and utilization of food during the 40 day period. Each group was restricted to 975 calories. Circles indicate failure to consume all of the food.

Chart 5

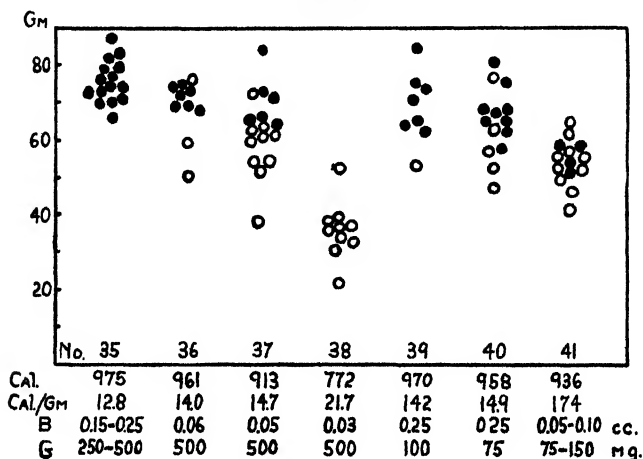


CHART 5. The effect of tikitiki (B) and of autoclaved liver (G) on the gain in weight and on the consumption and utilization of food during the 40 day period. Each group was restricted to 975 calories. Circles indicate failure to consume all of the food.

tite followed. These results agreed with those obtained on the higher caloric level (Chart 2). A supplement of 1.0 gm. of yeast daily (Group 25) was

not so satisfactory as the use of 0.225 to 0.5 gm. The rats in both 7 and 25 were slower in consuming the daily allowance of food and never appeared to be as hungry as those fed 0.5 gm. of yeast. Since even the 1365 caloric level represented considerably less food than would be consumed on a properly supplemented diet fed *ad libitum*, the results with a supplement of 1.0 gm. of yeast daily supported the conclusion stated in the first paper (1) that certain yeasts, at least, are decidedly inferior to combinations of tikitiki and autoclaved liver as a source of vitamins B and G.

Chart 6

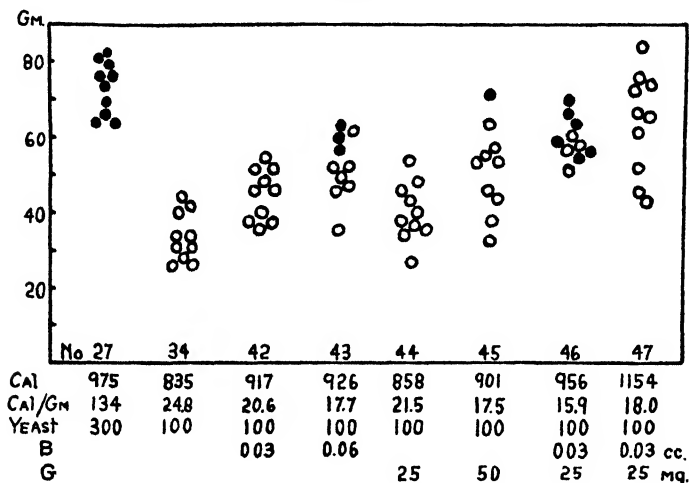


CHART 6. The effect of tikitiki (B) and of autoclaved liver (G) on the gain in weight and on the consumption and utilization of food during the 40 day period. Each group except Group 47 was restricted to 975 calories. Group 47 was restricted to 1365 calories. Circles indicate failure to consume all of the food.

Groups 35 to 38 (Chart 5) received an adequate supply of autoclaved liver and decreasing quantities of tikitiki. Groups 35, 39 and 40 show the effect of decreasing the autoclaved liver in supplements adequate with respect to tikitiki. The results were similar to those obtained with 1365 calories (Chart 3). As either B or G was lowered the gain in weight decreased. As in Chart 3 the principal effect appeared to be in the failure of the appetite. Group 41 represents the attempt to supply minimum quantities of B and G in order to duplicate, if possible, the results with yeast in which B and G were simultaneously reduced. The results were significant since comparison of 41 with 33 indicates that these two groups with practically the same caloric intake required approximately 17 calories per gram gain



in weight instead of the 13 calories required in optimum utilization. This result with 41 confirmed the previous observation that the efficiency of utilization of food is decreased if both B and G are inadequate.

Charts 6 and 7 present additional experiments in which the effects of tikitiki and of autoclaved liver on appetite were determined. The rats in Groups 34 and 42 to 47 received 100 mg. of yeast daily. This amount of yeast permitted very slow growth (34). The addition of 0.03 cc. and 0.06 cc. of tikitiki B daily slightly improved the appetite and growth but the same improvement resulted from supplementing the 100 mg. of yeast with

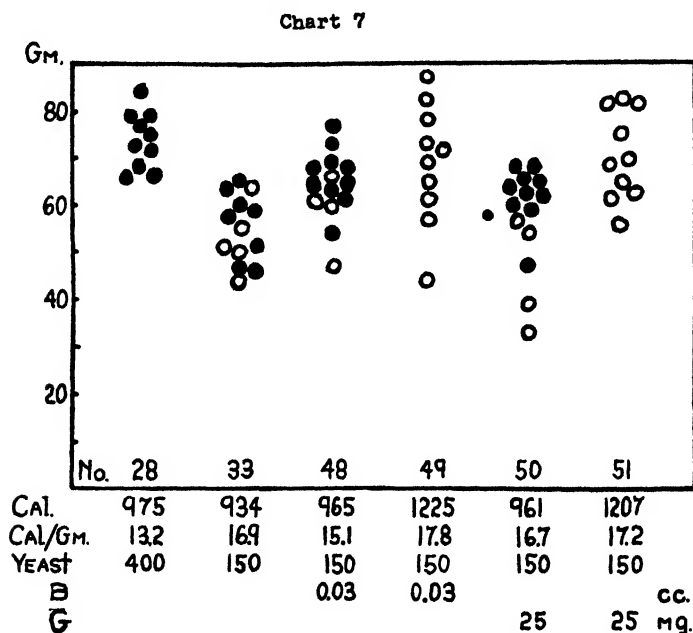


CHART 7. The effect of tikitiki (B) and of autoclaved liver (G) on the gain in weight and on the consumption and utilization of food during the 40 day period. Groups 28, 33, 48 and 50 were restricted to 975 calories. Groups 49 and 51 were restricted to 1365 calories. Circles indicate failure to consume all of the food.

25 and 50 mg. of autoclaved liver G. The results with 0.06 cc. of tikitiki and with 50 mg. of autoclaved liver respectively were inferior to the result obtained by supplying a combination of 0.03 cc. of tikitiki and 25 mg. of autoclaved liver. Chart 7 shows the effect of adding 0.03 cc. of tikitiki and 25 mg. of autoclaved liver to 150 mg. of yeast as supplements for groups on both the 975 and 1365 caloric levels. In neither case was the effect produced by tikitiki superior to that produced by autoclaved liver.

## DISCUSSION

Tikitiki and autoclaved liver, sources of B and G respectively, produced only moderate stimulation of appetite if added separately to supplements low in these two vitamins. The most marked increase in the consumption of food resulted from the addition of both tikitiki and autoclaved liver to the low B and G supplements. There was no evidence in these experiments that B was more necessary than G for the regulation of the appetite of young growing rats. The two vitamins not only appeared to be of equal importance in this respect but also seemed to depend upon the presence of each other for their activity. These data were obtained in experiments in which the appetite factors were concerned with maintenance and with slow growth. There was no apparent difference in the qualitative character of the appetite factors involved in the consumption of small amounts of food and in the optimum consumption of food (1) since slow growth or optimum growth could be produced at will by controlling the food consumption through variations in the amounts of tikitiki and autoclaved liver in the daily supplements. These experiments neither denied nor supported the assumption that the vitamin B complex consists of fractions other than B and G.

The efficiency of utilization of food by young rats was apparently unaffected by wide variations in the amounts of vitamins B and G in the daily supplements. Sources of these two vitamins producing only fair growth in *ad libitum* experiments in which appetite was a factor permitted optimum utilization if the food intake was restricted to a caloric level below that of the *ad libitum* experiments. However, utilization was affected if both B and G were simultaneously decreased to low levels. The fact that utilization was inferior was indicated by a smaller gain in weight even though all of the food was consumed. Utilization may also have been inferior on those supplements which were low in either B or G. In most of such experiments, however, appetite was decreased so that the rats could no longer be compared on the basis of equal caloric intake. It should be emphasized that these experiments were concerned with caloric levels which were considerably below the level of food intake in rats supplied an adequate diet *ad libitum*. The results were of interest in view of the observation of Palmer and Kennedy (10) that rats utilized adequate artificial diets as well as a mixture of natural food materials.

St. Julian and Heller (11) determined the coefficients of digestibility of protein, fat and carbohydrate on normal and deficient diets and found that the coefficients were unaffected even on diets low in B and G. This sug-

gested that the inferior utilization of food observed in our experiments on low B and G diets was connected with the metabolism of the ingested food. It was not possible to determine whether the decrease in efficiency was in the utilization of food for maintenance, for growth or for work. The importance of both vitamin B and vitamin G for the utilization and the consumption of food indicated that their metabolic functions might be closely related.

### SUMMARY

1. The influence of yeast and of combinations of tikitiki and autoclaved liver on the consumption and utilization of food in young rats have been determined in experiments in which the caloric intake was restricted to either 1365 or 975 calories during a 40 day experimental period.

2. Tikitiki and autoclaved liver, sources of vitamins B and G respectively, were found to be of equal importance as appetite stimulants.

3. The utilization of food (for growth) was found to be decreased on diets low in vitamin B and vitamin G.

4. It was concluded that vitamin G, as well as vitamin B, has an important part in the physiological mechanism controlling the consumption and the utilization of food.

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## Editorial Review

### THE EFFECT OF MUSCULAR EXERCISE ON THE METABOLISM OF ETHYL ALCOHOL

**A**LTHOUGH alcoholic beverages have been used by mankind for many centuries, it is surprising how little is absolutely known with regard to the utilization and metabolism of alcohol in the animal body. It is only within a decade or two that much advance has been made with regard to the fate of alcohol, particularly with regard to the concentrations of alcohol in the tissues and its disappearance from the tissues under various conditions. It is well known that in some countries, particularly in Europe, some groups of workers performing hard labor consume relatively large amounts of alcohol. It is therefore not only of academic interest, but of practical importance to know whether the metabolism of alcohol is accelerated by muscular exercise. If more alcohol is burned during muscular exercise than during rest and there is no serious impairment of efficiency, then alcohol is of value as a source of muscular energy, and it could be no longer contended that the energy for muscular activity was derived exclusively from any particular substance. If muscular exercise accelerates the combustion of alcohol, therapeutic use could be made of this fact when attempts are made to bring back an intoxicated individual to a normal condition or when one wishes to prevent the toxic and narcotic effects of large amounts of alcohol. If, on the other hand, there is no greater combustion of alcohol during muscular work than during the condition of rest, alcohol would occupy a unique place among the substances which can furnish appreciable amounts of energy in the animal body. Whatever may be the material, which is the immediate source of energy for muscular contraction, all classes of nutrients, protein and fat, as well as carbohydrates, are ultimately drawn upon to furnish directly or indirectly energy for muscular activity when there is an inadequate food supply. It is therefore of value both theoretically and practically to ascertain whether muscular activity accelerates the combustion and metabolism of alcohol.

The principal investigations on the effect of muscular activity on the metabolism of alcohol in men and animals are reviewed in this contribution.

Chauveau (7) made observations on the metabolism of a dog, varying 18 to 21 kilos, during a period of feeding, lasting over 389 days. The dog was made to run daily one or two hours in a rotary treadmill and his

respiratory exchange was measured at the same time. The respiratory exchange was also measured during periods of repose during the day, and during the periods of sleep at night. Shortly before the work began the dog was fed 500 grams of raw meat and 252 grams of sugar. From time to time there were substituted in this ration 48 grams of alcohol for 84 grams of sugar. The exercise varied from runs of 13.6 to 26.6 kilometers in the sugar experiments and from 9 to 21.1 kilometers in the experiments with alcohol, during approximately one to slightly over two hours. The average respiratory quotient in the four experiments without alcohol during the exercise periods was 0.963, whereas in the seven experiments in which alcohol was substituted, the respiratory quotient was 0.922. Chauveau calculated that the respiratory quotient should be 0.763 with 168 grams of sugar and 48 grams of alcohol if the respiratory quotient were 0.963 with 252 grams of sugar. The quotients found during the periods of repose were 0.904 and 0.871 as compared with a theoretical quotient of 0.716, when alcohol was substituted. The quotient for the total day was 0.921 on the days without alcohol and 0.885 instead of the theoretical 0.730 on the alcohol days. According to the author the enormous differences between the results found and those theoretically calculated show that when the organism is saturated with alcohol it is not able to utilize alcohol as a source of potential energy, either for the execution of the total physiological processes in the state of repose, or for the performance of work during muscular exercise.

The conclusions of Chauveau have been criticised by v. Hoogenhuyze and J. Nieuwenhuyse (22) for considering only the respiratory quotient and ignoring the lower carbon dioxide elimination and oxygen absorption during work on days with alcohol. They also consider the dose too disproportional (authors' word) to the body weight to be of practical value.

Atwater and Benedict (1) made several series of 24-hour experiments with humans in a respiration calorimeter. The balances of nitrogen, carbon, and energy were determined for each day. The available energy of the intake varied from 3502 to 3623 calories for one subject, and 3216 to 3276 calories for the other. The subjects worked 4 hours each morning and afternoon daily on a bicycle ergometer. In the first series the work equalled 158 to 211 calories, in the second 237 to 270 calories, and in the third series 127 to 277 calories per day. In the first series the diet on 4 days contained, beside protein, 129 grams of fat and 484.6 grams of carbohydrate, and then on 4 days the diet was 158.5 grams of fat, 296.1 grams of carbohydrate and 72.4 grams of alcohol. In the second series of experiments there were 9 days, 3 days with 106.0 grams of fat, 470.7 grams of carbohydrate, 3 days

with 104.2 grams of fat, 340.9 grams of carbohydrate, and 72.0 grams of alcohol, and then 3 days with 160.8 grams of fat and 342.7 grams of carbohydrate. The last series consisted of 9 days with two 3-day periods of fat and carbohydrate and one 3-day period in which 72 grams of alcohol replaced 122.9 grams of carbohydrate. The energy balances were negative on all but one of the days. In the 5 groups of days without alcohol the balances varied on the average from  $-329$  to  $-435$  calories. In the 3 groups with 72 grams of alcohol the balances were  $-313$ ,  $-262$ , and  $-405$  calories.

The authors drew the following conclusion: "That a part of the potential energy of the alcohol was transformed into the kinetic energy of muscular work these experiments do not prove, though they make it highly probable. They imply, that, so far as the utilization of the total energy of the diet was concerned, there was a slight advantage in economy in favor of the ordinary diet as compared with the alcohol diet, especially when the subjects were at hard muscular work, but the difference was inside the limits of experimental error and too small to be of practical consequence. On the average it was less than 1 per cent of the total energy and hardly reached 5 per cent of the energy of the alcohol. From this it follows that the energy of the alcohol was utilized nearly if not quite as well as that of the fats, sugar, and starch which it replaced."

This conclusion has been repeatedly cited in part or wholly by most of the investigators who have since studied in this field.

Grehan (10) determined by the Nicloux method the rate of disappearance of alcohol from the blood in dogs before and after the carrying out of muscular exercise. A dog was given by stomach tube 25 cc. of 10 per cent alcohol per kilo and was made to run in a rotary wheel 5.65 kilometers during one hour. Samples of blood were taken from the jugular vein one and two hours before the beginning of work and again at hourly intervals after the work was finished. The introduction of the alcohol took place at 11 to 11:15 a.m. and the first sample of blood was taken at 1:45 p.m. Between 3:45 and 4:45 p.m. the dog exercised and the drop in content of alcohol per 100 cubic centimeters was 0.05 cc. during the work as compared with 0.02 cc. and 0.04 cc. before the work and 0.035 after the work. In another experiment a dog was given 25 cc. of 10 per cent alcohol per kilo at 8 to 8:20 a.m. Samples were taken at hourly intervals beginning 5 hours later. The fall in blood alcohol was 0.029 cc. and 0.0285 cc. before the work, 0.055 during the work, and after the work ceased 0.009. The author concluded that muscular exercise favored the disappearance of alcohol but less than one supposed from *a priori* reasoning.

Durig (8) conducted observations on himself as to the influence of alcohol upon the respiratory exchange and performance of muscular work at high altitudes. The series consisted of 12 trips of mountain climbing with a load of 18 to 19 kilograms at an altitude of 2430 meters and in each of the 12 there were 4 successive periods of measurements. Four of the days with 16 periods were after the ingestion of alcohol in the morning and the alcohol days alternated with days on which no alcohol was taken. On the alcohol days the subject took 30 cc. of alcohol diluted with 125 to 250 cc. of water and sweetened with sugar at breakfast instead of the tea taken on other days. After camp duties were performed, which required one-quarter to one-half hour after taking alcohol, there was a walk of one hour to the place of the experiment. Then followed the four periods of work at approximately 800 kilogrammeters per minute. On the non-alcohol days the respiratory quotients in all 4 groups fell from the first period to a somewhat lower value by the fourth period: from 0.87 to 0.83, 0.86 to 0.84, 0.78 to 0.75, 0.81 to 0.76. On the alcohol days, on the contrary, the first quotients on two days were lower than any of the succeeding quotients, that is, there was a rise from 0.75 to 0.81 and from 0.82 to 0.86. On the other 2 days there was a change from 0.85 to 0.81 and no definite change in the fourth series. The author reasoned that if the alcohol were not burned in the preliminary walk, the respiratory quotient would have been lower during the experimental periods themselves because the alcohol would be burned at that time, and should be lower than the values on the non-alcohol days. Because of the fact that the quotient rose instead of falling on two days, he reasoned that the greater part of the alcohol was burned during the preliminary hour of walking, that gradually less alcohol was available for the performance of muscular work because of the disappearance of alcohol, and therefore the respiratory quotient would gradually rise during the four successive periods of work. In other words, the alcohol had practically disappeared during the preliminary hour of walking when no measurements were made, and, therefore, was utilized to supply part of the energy for the performance of muscular work.

These experiments have been cited by many authors, particularly with reference to another phase of the study, that is, the efficiency, but it is evident that they furnish but very little proof of any actual combustion of alcohol during muscular work. The evidence and reasoning is wholly indirect and in the light of today's knowledge it is questionable whether the alcohol would have been absorbed so fast and so completely during one and one-half hours that it would have been burned by the time the first period began. There is also the known effect of food which would ob-

scure the study with reference to the availability of alcohol for work. It is well known that the presence of food delays the absorption of alcohol so that it never rises to the same degree of concentration in the blood as it does when taken on an empty stomach. In addition to the sugar there was also ingested bread, so that there was food enough in the stomach to lower the absorption rate of alcohol.

Völtz and Baudrexel (24) determined the alcohol eliminated in the respiration and urine of a dog weighing 10 to 11 kilograms after the ingestion, at the beginning of the experimental day, of 1.7 to 1.9 cc. of alcohol per kilogram, i.e., 19.44 and 20 cc. of a 9.73 per cent solution by volume. In one series of observations the dog rested the entire day and the expired air was collected for 3 hours from a mask attached to the animal. The remainder of the day the dog was in a respiration chamber. In a second series there were 5 minutes of running, with 5 minutes pauses, with a total of 3 hours running and rest, during which the expired air was collected from a mask. The animal was in a respiration chamber the remainder of the day. The total run averaged 8.43 kilometers. In a third series, the dog ran 10 minutes, rested 5 minutes, for a total period of 3 hours with a run of 11.4 kilometers. In a fourth series the amount of exercise was the same, but the exercise did not begin until after a lapse of 3 hours after the alcohol ingestion. The per cent of the total ingested alcohol eliminated in the respiration in 3 hours averaged 0.75 per cent in the first series of four rest experiments; 4.27 per cent of the total in the series with  $1\frac{1}{2}$  hours' total exercise; 5.66 per cent of the total ingested alcohol in the series with 2 hours' work; and 3.68 per cent in the series with 2 hours of exercise when the exercise began 3 hours after the ingestion of alcohol. The total amount of alcohol eliminated in the respiration in 22 to  $23\frac{1}{2}$  hours in the same four series of experiments was as follows: first series, 2.02 per cent of the alcohol ingested; second series, 5.07 per cent; third series, 6.68 per cent; and fourth series, 4.82 per cent of the amount of alcohol ingested. Thus, exercise in a dog amounting to a run of over 11 kilometers in 2 hours' time resulted in a marked increase in the elimination of alcohol in the breath as compared to the amount eliminated when the animal was at rest. The reviewer considers that the possible part played by the elimination of alcohol in the breath during exercise, in decreasing the alcohol concentration in the body, must not be neglected in judging of the effect of exercise on the metabolism of alcohol.

Krieger (14) carried on a series of observations on himself with respect to the nitrogen balance as affected by muscular work with and without the addition of alcohol. The diet was so arranged that it was insufficient to



cover the daily needs with respect to the energy requirement and there would thus be always a nitrogen loss, particularly on the days on which there was muscular activity. The diet contained approximately 17.2 grams of nitrogen, 74 grams of fat, 177 grams of carbohydrate, with a total energy content of 1855 calories per day. There were five experimental periods. The first was a preliminary period of 5 days during which the diet was taken and the ordinary light activity of laboratory work carried on. This was followed by a period of 9 days without alcohol during which the subject rode a bicycle 50 kilometers a day. An intermediate period of 4 days followed in which the diet and muscular activity were the same as in the preliminary period. There was then a period of 9 days during which there was added to the daily diet 1600 cc. of wine containing 6.56 per cent of alcohol and the work was similar to the previous 9-day period. This period was followed by a period of 5 days which was similar to the preliminary period. It was estimated that the energy required to cover the work performed was equal to 729 calories and that this could be supplied by 104.1 grams of alcohol. The wine actually contained 105 grams. It was taken in 4 portions during the day.

There were average negative nitrogen balances in all of the periods with the exception of the work period of 9 days in which alcohol was taken in addition to the diet. Specifically, in the work period without alcohol, there was an average negative nitrogen balance of 1.86 grams per day. On the contrary, in the period with alcohol and work there was an average positive balance of 0.12 gram of nitrogen per day.

The author concluded that the alcohol became available for the performance of muscular work, as shown by the fact that the negative nitrogen balance without alcohol was changed to a plus nitrogen balance with alcohol. However, the average nitrogen intake was 16.36 grams per day in the work period without alcohol and 17.08 grams per day with alcohol, so that part of the shift from the negative balance to a positive balance may have been due to the slight increase in the food intake. This would render questionable whether the alcohol actually furnished energy for the performance of muscular work. It is doubtful whether experiments of this sort can be utilized to demonstrate the availability of alcohol for the performance of muscular work.

v. Hoogenhuyze and Nieuwenhuyse (22) determined the respiratory exchange by means of the Zuntz-Geppert apparatus before and after taking alcohol on rest days and on days during which muscular work was performed. The procedure was as follows: At 9 a.m. the subject reclined in an easy chair. A determination of the respiratory exchange was made and the

subject took 60 cc. of 90 per cent alcohol diluted with 90 cc. of water. After 10 minutes he breathed through the valves for another 10 minutes and then samples of air were collected for 6 minutes. An hour later another test was made and likewise at 3 p.m.

On the work days the program was as follows: At 9 a.m. the resting metabolism was determined with the subject sitting on a bicycle ergometer. He then pedalled for 20 minutes at the rate of 500 kilogrammeters per minute. Then, on the alcohol days, the same amount of alcohol was given as previously and immediately the pedalling was begun at the rate of 380 kilogrammeters per minute. The subject breathed for 15 minutes freely and then for 5 minutes through the valves and a sample was then taken. An hour later the exercise was again resumed and the expired air was again collected for analysis. The same process was repeated at 3 p.m. Apparently the subjects had both breakfast and lunch on the experimental days.

The average respiratory quotient of one subject was 0.87 on the resting day before taking the alcohol, 0.78 twenty minutes after the alcohol, and 0.75 one hour later. At 3 p.m., it was 0.86. The other subject had an average respiratory quotient of 0.91 before the alcohol and 0.72, 0.74, 0.78 after taking alcohol. On the non-alcohol days the drop in respiratory quotient either did not take place or was not nearly so marked.

On the days with muscular work, the average respiratory quotient with one subject was 0.82 during rest, 0.88 during work 20 minutes after alcohol, 0.84 at one hour, and 0.89 at 3 p.m. The other subject had a change from 0.85 during rest to 0.81, 0.78, with a rise to 0.85 at 3 p.m. Both the carbon dioxide and the oxygen values were lower after taking alcohol than in the corresponding periods without alcohol on the non-alcohol days.

Their conclusion was that alcohol directly or indirectly, not only produced energy for muscular exercise, but that the muscular exercise was performed more economically immediately after the taking of alcohol.

Mellanby (16) determined the alcohol in the blood of dogs at rest and before and after muscular activity during the course of 6 hours after the ingestion of 30 cc. and 50 cc. of alcohol. In the resting experiments the dogs were confined to a small cage and in the exercise experiments the animals were encouraged to run as vigorously as possible about in the open. When the larger quantities were given, the dogs ran behind a bicycle, but not to exhaustion, which is brought on easily in animals under the influence of alcohol. When the dogs received 4.1 cc. of alcohol per kilo, the results indicated that the alcohol was but slowly consumed, and that there was but little difference between the resting and active states. When the dogs were given 30 cc. of alcohol, or 2.5 cc. per kilogram of weight, the exercise in-

creased the rate of disappearance so that after exercise there was approximately 4 cc. less alcohol in the body than in the rest experiments, assuming that the concentration of alcohol in the blood was a measure of the total alcohol in the body. The results were even more pronounced when 20 cc., or 2.0 cc. per kilogram, were given. Roughly, the animal resting had 6 cc. more of alcohol in the body at the end of 6 hours than the animal working.

In another observation the dog ran about except for a rest of one hour after the alcohol had been ingested. Toward the end of the experiment there was also another rest period. During the rest periods the fall in concentration of the alcohol in the blood was less than during the work periods. As a possible explanation of the difference between the results with the higher concentrations and the lower concentrations, Mellanby suggested that the dogs were capable of doing more work at the lower concentrations because they were not under so great a toxic influence of the drug. They were more active, although it must be pointed out that even with the larger doses, notwithstanding greater fatigue, the dogs probably covered about 8 to 10 miles in the experimental period.

His general conclusion was that alcohol combustion underwent a more rapid rate during exercise than during rest when alcohol is present in low concentrations, but that at higher concentrations alcohol is oxidized at the same rate in the resting and active animal. That is, the greater the toxic action of the alcohol, the more restricted is the increase in the rate of combustion by exercise and the closer are the rates of combustion of alcohol in the active and resting states.

Viale and Gianturco (23) made observations on the physiological effects of alcohol at an altitude of 2900 meters and in Turin and Naples. They measured pulse, respiration, vital capacity, volume of expired air, and respiratory exchange before and after the ingestion of alcohol. Alcohol was taken as diluted brandy containing 28 cubic centimeters of alcohol. The respiratory exchange was measured by means of the Douglas bag method before and at 30-minute intervals after ingestion of alcohol. The work was climbing and descending 100 meters. They came to the conclusion that small doses of alcohol were useful because of their moderating influence on heart strain and on the respiratory mechanism, and that alcohol could also be used for the production of work and heat in the place of other materials, and that this was particularly evident on high mountains.

Miles (18, 19) determined the alcohol in the blood and urine of a human subject at intervals during the course of nearly 4 hours. In one experiment the subject exercised on a bicycle ergometer at the rate of 1.3 calories

external work per minute in 15-minute periods that alternated with periods of psychological tests. Three samples of blood were taken after the ingestion of 27.5 grams of alcohol in 1000 cc. of water and grape juice. These samples were drawn immediately after the work period while the subject was still sitting on the bicycle ergometer and urines were collected as soon as possible thereafter. On another day a similar experiment was carried out, but the subject rested the entire time with the exception of the activity which might have to do with the taking of the samples of urine and blood.

In the experiment with work the maximum concentration of alcohol in the urine was 0.42 milligram per cc. at 80 minutes after the ingestion and the maximum alcohol content of the blood was 0.20 milligram at approximately the same period of time. After 170 minutes had elapsed after the ingestion of the alcohol, the urine contained 0.33 milligram of alcohol per cc. and the blood 0.13 milligram per cc. In the experiment in which no muscular work was performed, at 80 to 82 minutes after ingestion the urine contained 0.44 milligram and the blood 0.28 milligram of alcohol per cc. and at 170 minutes after ingestion the urine had 0.29 milligram and the blood 0.13. The blood shows practically no greater change in alcohol concentration on the day with exercise than on the day at rest. The urine has almost identical values in both experiments, so that in Miles' experiments there is no evidence that the performance of muscular work after the taking of alcohol affects the concentration of alcohol in either blood or urine.

Sommerkamp (20) determined the course of the nitrogen excretion in two hour periods from 7 a.m. to 9 p.m. under conditions of fasting and rest and compared it with the course of the nitrogen elimination on other fasting days in which muscular exercise was performed between 11 a.m. and 1 p.m. The exercise consisted of either 25 kilometers of bicycling or 5 kilometers of rowing during two hours. On two days the same amount and kind of work was performed, but at the beginning of the work, 70 grams of dextrose in 200 cc. of water were taken in one case, and 100 grams of dextrose in the other. Similarly, with the same kind and amount of work, 50 cc. of alcohol diluted to 200 cc. in volume were ingested on one day, and 76 cc. of 90 per cent alcohol diluted to 200 cc. were ingested on another.

The nitrogen excretion on the 5 days in which muscular work was performed without food fell from an average of 1.29 grams per 2 hours before work to an average of 0.76 gram per 2 hours at the end of the work period. The excretion on the 2 days with the dextrose fell from an average of 1.19

grams per 2 hours before work to an average of 0.81 gram per 2 hours at the end of the work period. Following the period of work on the days on which no sugar or alcohol was taken, there was a rise after the work period to higher levels, ending at the period from 5 to 7 p.m. between 0.68 and 1.13 grams per two hours. However, on the days with dextrose during the interval from 5 to 7 p.m., the nitrogen elimination for 2 hours was 0.69 and 0.80 gram. Thus, the nitrogen elimination did not rise on the day on which dextrose was given after the work as it did on the day when no nutrients were ingested.

On the two days with alcohol, the nitrogen elimination in the period from 7 to 9 a.m. averaged 1.28 grams. During the period from 11 a.m. to 1 p.m. the nitrogen elimination was 1.05 grams. Thus, during the work period on the alcohol days there was a greater elimination of nitrogen than on any other days, but following the period of work there was a marked drop for the next two periods in the nitrogen elimination, in all cases below 0.70 per 2 hours, and in the periods from 5 to 7 p.m. there was as low a value as 0.42. Thus, the drop in the nitrogen elimination on the alcohol days was even greater than on the days on which dextrose was given, and also greater than on the days on which no nutrients were ingested and work was performed.

The author concludes from his experiments that alcohol not only spares carbohydrate and fat, but that its energy is directly available for the performance of muscular work and that this is the first proof for it, which might be considered free from criticism.

Brechmann (2) made a study of the respiratory exchange with respect to the availability of alcohol for the supply of energy for muscular work. The experiments were carried out by the author on himself and the respiratory exchange was measured by means of the Zuntz-Geppert apparatus. The work was performed on a hand friction ergometer. The procedure of the experiment was as follows: The subject took breakfast which consisted mostly of carbohydrate. At 12 o'clock noon the respiratory quotient was determined. Then between 1:00 and 1:30 p.m. the midday meal was taken, consisting principally of carbohydrate. This was followed immediately by the ingestion of 60 grams of fructose. At 3:25 p.m., 40 grams of fructose were taken. The respiratory quotients were measured in the rest experiments at 2:15, 3:15, 4:00, and 5:30 p.m. On the days on which alcohol was given, 30 grams were taken at 2:45 p.m.

In the rest experiments without alcohol, the respiratory quotients were all practically at unity or over, with the exception of the first quotient on one day which was 0.71. On the rest days on which alcohol was taken, the

quotient at 3:10 p.m. in one case was 0.86, a drop from 1.04, and in the other case, 0.71, a drop from 0.99.

On the work days the work was performed from 11:45 to 12 noon, with a total amount of 3900 kilogrammeters. On the day without alcohol there was a rise from the respiratory quotient of 1.09 at 12 noon to 1.23 at 2:15 and 3:20 p.m. On the alcohol day, however, the quotient at 3:15 p.m. was 0.88, a drop from 1.16 on the measurement preceding at 2:15. Subsequently two series were carried out in which work was performed to the extent of 3600 kilogrammeters immediately preceding each measurement of the respiratory quotient. On the day without alcohol there was practically no change in the respiratory quotients throughout the series. On the day with alcohol the quotient at 2:25 p.m. immediately after work before taking alcohol was 1.10, whereas, at 3:30 p.m. it was 0.87 after the alcohol was taken.

The author came to the conclusion that the alcohol served as a source of energy for the performance of muscular work. However, the experiments are not conclusive to the reviewer because of the fact that the respiratory exchange was not measured during the work itself. There is no indication that the alcohol was utilized during the period of work. It is questionable whether a plan of investigation such as was carried out by Brechmann can contribute much to the question of the utilization of alcohol for the performance of muscular work.

Brechmann (3) made a further series of observations on the effect of ingestion of alcohol on the respiratory exchange during work and during rest. The routine of the experiments was as follows: The subject had breakfast at 9 a.m., consisting of coffee, milk and sugar, and zwieback. At 11:15 a.m. the principal meal of the day, consisting of noodles with sugar, potatoes, and bananas, was taken. The measurements began at 12 noon and lasted six hours. At 1:15 and 3:30 p.m. 60 grams of fructose were ingested. On a second day the same procedure was used, but at 2:25 and 3:25 p.m. 28 grams of alcohol in the form of 60 cc. of brandy were ingested. The work periods lasted as a rule 20 minutes with approximately 9000 kilogrammeters being performed. The measurements of the respiratory exchange during the work began after the subject had worked 10 to 15 minutes and usually it was measured for 5 minutes.

In all of the work periods, both on the days with and without alcohol, the respiratory quotients were lower than in the rest periods immediately preceding. For example, on the first day without alcohol, the respiratory quotient at 2:45 p.m. at rest was 0.97, at 3:20 p.m. during work it was 0.90. On the alcohol days the change at the same period of time was from

0.94 to 0.78 and at 4:15 and 4:40 p.m. the change was from 0.80 at rest to 0.74 during work. A similar series with dextrose showed always the same phenomenon, that is, a fall in respiratory quotient during the work period that was greater on the day with alcohol than on the day without alcohol.

The author explained the lowering of the respiratory quotient during work on the days without alcohol as due to a carbon dioxide retention in the tissues. The author concludes that the alcohol was available for the performance of muscular work and that more of it is burned during work than during rest. The results in this investigation are so at variance with other investigations with respect to the fall in respiratory quotients during the work periods that the reviewer is inclined to question their significance. In addition the first respiratory quotients measured at 12 noon seem low for so high a carbohydrate diet as was taken at 9:00 and 11:15 a.m. These respiratory quotients ranged on 6 days from 0.76 to 0.97. Excluding the highest value, the range is at the upper limit to 0.86. These quotients are therefore no higher than one would obtain frequently with a subject who has fasted for 12 hours, and these facts together with the invariable lowering of the respiratory quotient during work periods render the results doubtful. The rate of muscular work was about 450 kilogrammeters per minute and it has been our experience as well as that of others that the respiratory quotient during the first 20 minutes at this rate of work is raised from the resting value instead of lowered.

Galamini (9) determined by a modification of the method of Widmark every half hour the alcohol content of the blood at rest and during muscular work when alcohol was given at the rate of 0.5 cc. per kilo and diluted with an equal part of water. The alcohol solution was taken on an empty stomach just before beginning the work, which consisted of ascending and descending stairs, or in walking at a rapid pace in the streets of Rome. The alcohol in the blood at rest was as follows: 0.53 at 70 minutes, 0.55 at 25 minutes, 0.56 at 15 minutes and 0.51 milligram per cc. of blood at 45 minutes. When alcohol was ingested at the beginning of work, the alcohol in the blood was 0.26 in 60 minutes, 0.24 in 45 minutes and 0.32 milligram per cc. in 65 minutes. In spite of the lower figures during muscular work, the author concluded that because the glucose content of the blood after the ingestion of cane sugar was not appreciably altered by the ingestion simultaneously of alcohol, it was a question whether the alcohol was used during muscular work. He found practically the same increase in the sugar of the blood during muscular work whether cane sugar was given with or without alcohol and concluded that further experiments were necessary

with measurements of the respiratory exchange in order to settle the question.

Carpenter (4) exposed hens to alcohol vapor in a chamber for periods of 2 to 27½ hours. The animals were confined in a wire cage which was supported in such a manner that records of the activity or lack of activity were made upon a long paper kymograph during the entire time of exposure. The largest group was exposed from 15½ to 16½ hours individually. It was found that at the extreme limits of concentration of alcohol in the tissues, namely, from 0.29 to 2.27 milligrams of alcohol per gram of tissue, there was an inverse relationship between the activity and the concentration of alcohol in the whole body, that is, the more active the animals, the less was the alcohol in the body at the end of the exposure. This was most marked at the extremes and did not follow necessarily in the order of graded activity. In order to produce artificial activity, the hens were stimulated by periodic shocks from an induction coil. These hens, however, showed about the same range of alcohol concentrations as the group in which the hens were free to respond according to their own condition. In general, the most active hens in the group were lowest in concentration of alcohol, whereas the less active had the highest amount at the end of the exposure. With a group of 6 hens exposed repeatedly to alcohol vapor, a direct rather than inverse relation seemed to exist between activity and alcohol concentration in the tissues.

By the method of exposure it would seem as though the greater the activity the greater would be the absorption of alcohol, because activity resulted in an increased respiration, which automatically would bring a greater amount of alcohol into the lungs to be absorbed from the inspired air. These experiments as a whole indicate that the effect of activity was to prevent the accumulation of high concentrations of alcohol. The evidence is entirely indirect and unfortunately no comparisons were made between the total metabolism during exposure and the concentration of alcohol in the tissues.

Terroine and Bonnet (21) made a study on the effect of the ingestion of alcohol on the heat production of men, pigeons, and rabbits, in the conditions of thermic neutrality with men and in the conditions of both thermic neutrality and at temperatures at which an extra heat production was required on the part of the animals. The men received 400 to 600 cc. of white wine containing 10.8 per cent of alcohol. Their basal metabolism was first measured in 15-minute periods by means of a mask and a Tissot spirometer. After the ingestion of the alcohol, the respiratory exchange was measured for over 8 hours. From the respiratory exchange was computed



the extra heat which was produced and this was compared with the potential energy of the alcohol. In one case, the extra heat equalled 94.3 per cent, in the other case, 92.3 per cent of the potential energy of the alcohol ingested.

With the rabbits, the alcohol was introduced intraperitoneally. With the pigeons it was introduced by intramuscular injection or by the mouth. The heat production of the pigeons at 29° was 6.5 calories per kilogram per hour before ingestion. After ingestion it was 8.4 and 8.6 and the total extra heat amounted to 94 and 95.5 per cent of the potential energy of the alcohol actually absorbed by the animals. At 12° the heat production of the pigeons before ingestion averaged approximately 8.6 calories and after ingestion the heat production varied from 8.3 to 8.9 calories per hour per kilogram. Thus, there was practically no change in heat production at 12° as the result of ingestion of alcohol. In 2 experiments with rabbits the extra heat production was equal to 96 and 97.4 per cent of the calorific value of the alcohol absorbed.

The authors concluded that at thermic neutrality the energy from alcohol can not take the place of energy required for synthesis or growth, or for external muscular work, that is, that it burns without any profit to the organism. On the contrary, when there is a cause for extra heat production as at low temperatures, alcohol can furnish heat. Therefore, the nutritive value of alcohol consists in conversion into heat only and alcohol can not be utilized in cellular transformations of energy or in the performance of muscular work.

The results of these experiments are quite at variance with the results found by other workers with respect to the extra heat production as the result of the ingestion of alcohol. There were astounding increases in heat production in individual periods with the human subjects, for example, from 17.13 calories per 15 minutes before alcohol ingestion to 35.75 calories in 15 minutes, an increase of over 100 per cent. The study, however, is highly suggestive with regard to a method of approach to a solution of the question of the nutritive value of alcohol.

Cassinis and Bracaloni (6) determined the alcohol in the blood and the urine of men at intervals after giving 0.5 cubic centimeter of alcohol per kilo, actually from 30 to 35 cc., diluted with a double volume of water. A series of measurements with the subjects at rest showed maximum concentrations of alcohol in the blood of 0.76 milligram per cubic centimeter at 41 minutes in one subject, 0.565 at 50 minutes and 0.73 at 40 minutes in others. With a run of 3600 meters (presumably on a level grade) at the 29th to 55th minute after ingestion, the maximum concentration of al-

cohol was 0.42 milligram at 48 minutes. With a run of 3600 meters from the first to the 25th minute, the maximum concentration of alcohol was 0.35 at 28 minutes, but subsequently at the 86th minute it was 0.48 milligram per cc. of blood. With a march of 9600 meters from the 17th to the 110th minute, the maximum concentration was 0.466 at 65 minutes. With stationary rowing from the 13th to the 63rd minute, there was a maximum concentration of 0.559 at 30 minutes, and subsequently a fall to a value of 0.29 at 115 minutes. Thus, muscular work in these cases produced a noticeable effect in preventing a rise in the percentage of alcohol in the blood to the same extent that occurred during rest.

Meyer (17) determined by the Douglas bag method the respiratory exchange of a man of 64 kilograms accustomed to large quantities of alcohol. This subject took 240 cc. of absolute alcohol in the form of beer and whiskey and food, consisting of 2 sausages on buttered bread in the course of two hours and was not affected enough to prevent the performance of muscular work on a bicycle ergometer. The respiratory exchange was measured after 5, 20, 40, and 50 minutes of work at 700 kilogrammeters per minute. One series of experiments was immediately after taking the alcohol, another followed two hours after the taking of the alcohol, and a third 12 hours after the ingestion of the alcohol. The main object of the study was to determine the efficiency but the respiratory quotients are given as averages for the measurements of one hour. With alcohol immediately preceding the work, the average respiratory quotient was 0.88 in four experiments. In 8 control experiments it was 0.868. When the alcohol was taken 2 hours before work, the average respiratory quotient during the work was 0.89. When the alcohol was taken 12 hours before the work, the average respiratory quotient during the work was 0.853 as compared with 0.87 in the control experiments. The author pointed out that the respiratory quotients show that the combustion of the alcohol was extremely slow and that with this subject who was accustomed to large amounts of alcohol, the rapidity of burning was no greater, and in fact, not so great as has been found in earlier studies with subjects unaccustomed to alcohol.

These experiments seem extraordinary because of the ability of a man to carry on muscular work after the consumption of 240 cc. of alcohol (3.75 cc. per kilogram) and the apparent lack of combustion of alcohol during the work. The first series of experiments was undoubtedly too soon after the consumption of the alcohol in order for its full effect to have taken place, but two hours after the ingestion of alcohol one would expect that enough had been absorbed to furnish an appreciable amount for the metab-

olism for muscular work. Similarly, at the end of 12 hours with this amount of alcohol, there must have remained in the body a considerable amount so that it was still available for the performance of muscular work. It is difficult to conceive of the disappearance and combustion of this amount of alcohol in the body without affecting the respiratory quotients.

Hitchcock and Kraft (13) in a preliminary report at the annual meeting of the American Physiological Society stated that the respiratory quotients during rest, work, and recovery were slightly depressed by the ingestion of small amounts of ethyl alcohol. The respiratory quotient of the excess metabolism of work was lower after taking alcohol than in control tests. This suggested to them that the alcohol furnished part of the energy used by the muscles in doing work.

A survey of the preceding abstracts will show that the majority of the investigators believe that their results indicate that alcohol is utilized or burned in muscular work. Those who have come to the conclusion that their work disproves or minimizes the availability of alcohol for muscular activity are in the minority. They are Chauveau (7), Terroine and Bonnet (21), Galamini (9) and Meyer (17). However, none of these can be considered as critical experiments. The dose in Chauveau's experiments was large for normal performance and the work began too soon after the alcohol was taken. The work of Terroine and Bonnet was not a direct attack on the effect of muscular work itself on the metabolism of alcohol, and their results are so at variance with all preceding work on the effect of alcohol on metabolism that they must be considered with a good deal of reserve. Their observations, however, are distinctly challenging, and experiments of this type should be made as their method of study is a logical one. The observations of Galamini were relatively few and the time relationships were so irregular that it is difficult to compare the results under the conditions at rest with those at work. In fact, the few results that the author has given would indicate that muscular work prevented the rise in alcohol in the blood that occurred at rest and thus alcohol was more rapidly burned during work than during rest. Meyer's results on the respiratory quotient after the ingestion of 240 cc. of absolute alcohol are simply astounding and would indicate that there is a possibility of large quantities of alcohol preventing its combustion rather than accelerating it.

On the positive side the studies may be classified according to nitrogen balance and excretion, respiratory exchange, and alcohol in the blood. The studies on the nitrogen excretion may be considered as of the least importance and of the least significance. Nitrogen excretion is bound up with so many factors, such as accuracy of determination of nitrogen intake,

digestibility of food nutrients, diuresis, nitrogen in feces, nitrogen in sensible perspiration, and the maintenance of exactly the same amount of occasional muscular activity other than that due to the prescribed exercise, that it is questionable whether it would be possible to demonstrate by such a study whether muscular activity accelerated the rate of combustion of alcohol. Consequently the reviewer does not believe that the investigations of Krieger (14) and Sommerkamp (20) are decisive.

Of the studies in which the respiratory exchange was used as the criterion, three (v. Hoogenhuyze and Nieuwenhuyse, (22), Brechmann, (2), (3)) were made with the Zuntz-Geppert gas-analysis apparatus and the reviewer does not believe that the Zuntz-Geppert apparatus is in general suitable for such critical work as the differentiation between the amount of combustion of a substance in rest and work. Furthermore, all three of these studies were additionally complicated by the taking of two meals during the course of the measurements. The reviewer does not wish to imply that the results do not indicate that alcohol was burned, for the respiratory quotients in all three studies showed that alcohol depressed the quotient both during rest and either immediately following or during work. But it is impossible to draw the conclusion that muscular work accelerated the combustion of alcohol as the data are insufficient to apportion the metabolism among protein, carbohydrate, fat, and alcohol. The results simply indicate that after alcohol is ingested the combustion was rapid enough both during rest and work to cause a fall in the respiratory quotient. Probably no student of metabolism would deny that alcohol is burned in the animal body, at least to the extent of 90 per cent of the amount ingested. We know of no other means of its disposal at the present time. The investigation of Durig (8) can not be considered as decisive because of the admixture of food with the ingestion of alcohol. The preliminary report of the investigation of Hitchcock and Kraft (13) lacks details, so no critical assessment can be made at this time. When the respiratory exchange is used as a criterion of the influence of muscular activity on the metabolism of alcohol, it is necessary to have a method of measurement which can be considered free from possible objections such as questions of normality of breathing, method of measuring total metabolism, analysis of gases and methods of sampling. The respiratory exchange should be measured over the same period during rest as during work and the measurements in the muscular work experiments should include the recovery period as well as the periods of muscular activity. Control experiments both during rest and activity of the same duration and intensity of work are absolutely necessary to calculate the amount of alcohol burned when the respiratory ex-

change is used as a criterion. Alcohol has an effect on the subject, at rest at least, which tends to obscure the significance of respiratory quotients obtained following its ingestion. A subject tends to become drowsy and very easily drops off to sleep if efforts are not made either on the part of the subject himself or on the part of the observer to keep him awake. It is well known that drowsiness or sleep tends to cause the respiratory quotient to fall and that the breathing is irregular during the transition period from the condition of being alert and awake to the condition of sound sleep. Thus the interpretation of the respiratory quotients obtained under these conditions is attended with difficulties. There is also to be considered the physiological effect of alcohol on the sensitivity of the respiratory center and on the acid-base equilibrium and reaction of the blood. Higgins (11) found that alcohol was either without action on the respiratory center or that it sometimes acted to increase the sensitivity of the respiratory center as indicated by a drop in the carbon dioxide tension of the alveolar air. Himwich and co-workers (12) have reported recently on the effect of alcohol on the lactic acid and  $\text{CO}_2$  content of the blood and call attention to the necessity of taking into consideration the rapid shifts of the acid-base equilibrium in the interpretation of respiratory quotients after the ingestion of alcohol. Even when all of these conditions are met, there is still the question of what nutrients alcohol replaces and in what proportions. We do not know whether alcohol diminishes the metabolism of protein, fat, and carbohydrate according to the proportions existing before the alcohol was ingested, or replaces one substance more than the others.

In considering the third criterion of metabolism (combustion) of alcohol, namely, the alcohol content of blood or tissues, it is found that all but one (Miles, 18) show that muscular activity affects the concentration of alcohol in the blood or tissues in such a way that after muscular activity, the concentration of alcohol is lower than when the subject has rested the entire period under observation after the ingestion of alcohol. Miles had only one experiment under each condition and his dose was combined with 300 cc. of unfermented grape juice. One does not know whether the grape juice would prevent the combustion of alcohol in muscular work or not, but these two experiments can not be considered entirely free from possible objection because of the presence of readily oxidizable material in the grape juice. Miles' main problem was not the effect of muscular activity on alcohol concentration in the tissues but the comparison of alcohol concentrations in the blood and urine. Carpenter's (5) study was indirectly indicative and depended so much upon a qualitative assessment of a graphic record of activity that it can not be considered rigorously decisive.

The experiments of Grehant (10) and Mellanby (16) with dogs are both consistent in indicating that muscular exercise promotes the more rapid disappearance (presumably combustion) of alcohol from the tissues. There is an inconsistency in that with the higher amounts in Mellanby's experiments, there was no apparent effect on the alcohol concentration in the blood as the result of exercise. The studies of Galamini (9) and Cassinis and Bracaloni (6) on men also appear to be consistent with the general thesis that exercise accelerates the combustion or disappearance of alcohol in the body. The reviewer is of the opinion that there is a discrepancy between the calculation of the amounts of alcohol metabolized when the respiratory exchange is used as a basis for the computation, and when the alcohol in the tissues is used as a basis. Carpenter (4) called attention to the discrepancy in the time relationships between the metabolism of alcohol as indicated by the respiratory exchange and as indicated by the concentration of alcohol in the urine, and later (5) to the discrepancy between the alcohol that could be accounted for by metabolism and concentration of alcohol in the tissues of hens, and that actually absorbed during the exposure to alcohol vapor. In both groups of experiments the measurements of respiratory exchange and alcohol in urine of men and of absorption and alcohol content of tissues in hens were not made in each case on one and the same day, and therefore lack the unity that experiments of this kind would have if all classes of observations were made in the same experiment. Experiments are needed in which more than one of the criteria are used as a basis of interpretation. The alcohol content of the blood is undoubtedly the best method of determining the rate of disappearance of alcohol when the observation lasts until all alcohol has disappeared from the blood. The determination should be supplemented by the determination of the alcohol in the expired air and in the urine in order to have additional information on the paths of alcohol elimination. In fact, when it is not feasible to obtain samples of blood frequently, the alcohol in expired air can be used as an index of the changes in the blood, for Liljestrand and Linde (15) have shown that the alcohol in the blood and expired air undergo parallel changes at rest, and similarly, Miles (18) has shown that the alcohol in blood and urine are parallel at rest and during work, but not identical in concentration. The amount of alcohol in the expired air should be known because of the greater respiratory ventilation during work than during rest. The quantity of alcohol eliminated in the expired air might be of significance in lowering the alcohol content of the body. The best type of study of the metabolism of alcohol at rest and at work would therefore include the measurements of the respiratory exchange and the alcohol in

the blood, urine, and expired air as frequently as feasible continued until the alcohol has disappeared from the tissues. The reviewer is of the opinion that the literature by no means furnishes adequate data to enable one to draw a decisive conclusion in regard to whether or no muscular activity accelerates the combustion of alcohol. Although some of the previous work on the metabolism of alcohol during muscular work has been under the condition of accompanying ingestion of food, the experiments were not carried out for the purpose of determining the effect of food ingestion on the metabolism. The field of the study of the effect of food ingestion on the metabolism of alcohol during work has not been investigated, consequently there remains much further work to be done on the metabolism of alcohol as affected by muscular activity, either when given alone or in combination with various classes of nutrients.

T. M. C.

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## EFFECT OF DIET ON EGG COMPOSITION

II. MORTALITY OF EMBRYOS IN EGGS FROM HENS ON DIETS  
CONTAINING PROTEIN SUPPLEMENTS OF  
DIFFERENT ORIGIN

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## INTRODUCTION

BYERLY, Titus, and Ellis (19) found that eggs from hens fed different diets varied in hatchability and in the time of mortality of the contained embryos. Of the diets used, the ones containing only feedstuffs of vegetable origin yielded eggs in which a relatively large percentage of the embryos died during the second week of incubation. This high second week mortality was associated with a high incidence of chondrodystrophy, a type of defective cartilage and bone development described in chick embryos by Dunn (5) and Landauer (10). The experiments in the present paper extend and confirm the earlier report on the effect of diet on the ability of eggs produced to sustain embryonic life through the second week of incubation.

## MATERIALS AND METHODS

The data to be presented were collected during the period between September, 1929, and August, 1932, inclusive. Data were gathered on time of embryonic mortality in the eggs from the breeding flocks at Beltsville, Maryland, during the breeding seasons of 1930, 1931, and 1932. The breeding flocks were housed in lots of 10 to 25 females and a single male in colony laying houses situated on plots of ground from about 900 sq. m. to about 2,000 sq. m. in area. The plots contained a variable amount of blue-grass



TABLE  
EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSTROPHY IN EGGS FROM  
PRESENTED FOR

Diet	Birds	Fertile eggs set	First week		Second week	
			Mortality	Difference normal and other diets	Mortality	Difference normal and other diets
	No.	No.	Per cent	Per cent	Per cent	Per cent
Basal. ....	130	2563	10.3±0.70	+0.7±0.92	13.5±1.16	+10.9±1.21
Cottonseed meal..	46	698	12.6±1.14	+3.0±1.28	17.2±1.96	+14.6±1.99
Soybean meal. ...	77	2359	13.4±0.91	+3.8±1.13	18.7±1.65	+16.1±1.68
Ground soy beans.	38	1587	9.3±0.91	-0.3±1.13	11.0±1.47	+8.4±1.50
Yeast preparation.	32	500	5.8±0.81	-3.8±1.01	6.0±1.09	+3.4±1.14
Meat meal. ....	65	1241	15.2±1.31	+5.6±1.44	10.7±1.20	+8.1±1.24
Menhaden fish meal. ....	13	262	6.7±1.65	-2.9±1.75	13.3±3.06	+10.7±3.08
North Atlantic fish meal. ....	47	840	14.4±1.40	+4.8±1.52	3.2±0.78	+0.6±0.84
Crabscrap meal...	47	962	9.2±0.81	-0.4±1.00	2.8±0.45	+0.2±0.55
Buttermilk. ....	30	601	8.6±0.75	-1.0±1.06	3.6±0.63	+1.0±0.71
Normal. ....	111	4898	9.6±0.59	—	2.6±0.32	—
Breeding flocks Reds and Leg- horns only...	423	8752	10.1±0.39	—	2.3±0.15	—
Breeding flocks total. ....	1156	23880	11.4±0.25	—	2.6±0.11	—

TABLE  
EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSTROPHY IN EGGS FROM YEARLING  
IN CONFINEMENT AND

Diet	Birds	Fertile eggs set	First week		Second week	
			Mortality	Difference pullet year diet and others	Mortality	Difference pullet year diet and others
	No.	No.	Per cent	Per cent	Per cent	Per cent
Pullet year: various diets. ....	90	1850	22.3±1.56	—	30.5±1.36	—
Soy bean-cotton- seed. ....	10	158	20.7±5.10	-1.6±5.33	37.6±7.62	+7.1±7.74
Basal. ....	42	2513	17.1±1.89	-5.2±2.45	17.4±1.72	-13.1±2.19
Normal. ....	25	1007	11.5±1.32	-10.8±2.56	4.3±0.82	-26.2±1.59

## I

PULLETS ON VARIOUS DIETS WHEN FED IN CONFINEMENT; BREEDING FLOCK DATA  
COMPARISON

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference normal and other diets	Total	Difference normal and other diets	Incidence	Difference normal and others diets
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
17.2±0.84	-0.9±1.14	59.0±1.62	-10.7±1.86	7.45±0.84	+7.23±0.85
15.1±1.50	-3.0±1.68	55.1±2.81	-14.6±2.95	6.06±1.13	+5.84±1.13
18.5±0.98	+0.4±1.25	49.4±1.90	-20.3±2.21	5.05±0.56	+4.83±0.57
20.2±1.43	+2.1±1.62	59.5±2.59	-10.2±2.84	1.61±0.39	+1.39±0.40
18.0±1.54	-0.1±1.72	70.0±2.38	+0.3±2.74	1.60±0.52	+1.38±0.53
16.6±0.92	+1.5±1.20	57.5±2.21	-12.2±2.39	2.82±0.50	+2.60±0.51
30.7±3.72	+12.6±3.80	49.3±4.19	-20.4±4.29	2.00±0.78	+1.78±0.79
16.3±1.07	-1.8±1.33	66.1±1.87	-3.6±2.08	0.30±0.12	+0.08±0.14
15.9±1.14	-2.2±1.38	72.1±1.90	+2.4±2.21	0.13±0.087	-0.09±0.12
13.1±1.62	-5.0±1.79	74.7±1.86	+5.0±2.07	0.17±0.11	-0.05±0.13
18.1±0.77	—	69.7±0.91	—	0.22±0.074	—
13.3±0.39	—	74.3±0.60	—	0.017±0.105	—
17.6±0.30	—	68.4±0.42	—	0.28±0.039	—

## II

HENS WHICH PRODUCED CHONDRODYSTROPHIC EMBRYOS DURING THEIR PULLET YEAR, KEPT  
FED VARIOUS DIETS

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference pullet year diet and others	Total	Difference pullet year diet and others	Incidence	Difference pullet year diet and others
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
15.2±0.80	—	32.0±1.12	—	16.9 ±1.05	—
18.0±3.27	+2.8±3.36	24.0±7.82	-8.0±7.90	20.2 ±5.90	+3.3±5.99
13.8±1.00	-1.4±1.28	51.7±2.50	+19.7±2.74	1.68±0.37	-15.22±1.11
26.9±2.52	-11.7±2.64	57.3±2.86	+25.3±3.07	0.88±0.72	-16.02±1.13

TABLE

EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSSTROPHY IN EGGS FROM

Diet	Birds	Fertile eggs set	First week		Second week	
			Mortality	Difference confined birds on basal diet and others	Mortality	Difference confined birds on basal diet and others
	No.	No.	Per cent	Per cent	Per cent	Per cent
Basal.....	41	875	6.5±0.80	-3.8±1.06	0.6±0.20	-12.9±1.18
Soybean meal. . .	42	832	7.1±0.91	-3.2±1.15	2.8±0.87	-10.7±1.45
Linseed meal. . .	41	767	6.4±0.81	-3.9±1.07	1.3±0.35	-12.2±1.21
Peanut meal . . .	38	696	4.3±0.74	-6.0±1.01	2.4±0.79	-11.1±1.41
Breeding flock						
Giant-Leghorn						
F <sub>1</sub> only.....	109	1465	7.6±0.54	—	1.2±0.26	—

sod and were partially shaded. The breeding of the birds and evidence of genetic causes for differences within the breeding flock in time of embryonic mortality have been discussed by Byerly, Knox and Jull (3). The diet given the breeding flocks contained whole corn, wheat, and a small amount of semi-solid buttermilk in addition to the constituents of the normal diet fed confined birds. Eggs were incubated from about February 15 to May 1. These data are introduced to furnish a normal mortality curve for basic comparisons and to demonstrate the adequacy of the normal diet used for the confined birds to enable the production of eggs capable of sustaining embryonic life through the second week of incubation in normal fashion.

Portions of these breeding flock data have been statistically treated and included in Tables I and III for the same purpose.

The conditions of housing, management, and selection of experimental material for the experiments with confined birds on the "normal" diet and the composition of the diet have been described in some detail by Byerly, Titus, and Ellis (2).

The data on confined birds contained in this paper were obtained from experiments begun in September, 1929, 1930, and 1931. Each experiment covered a period of eleven months and some or all of the eggs produced each month were incubated. Pens of Single Comb Rhode Island Red and Single Comb White Leghorn pullets were used except in the experiment described in Table II for which Single Comb Rhode Island Red yearlings which produced chondrodystrophic embryos during their pullet year on experimental diet were used.

## III

## PULLETS FED ALL-PLANT SOURCE DIET, WITH ACCESS TO BLUE-GRASS RANGES

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference confined birds on basal diet and others	Total	Difference confined birds on basal diet and others	Incidence	Difference confined birds on basal diet and others
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
15.0±1.16	-2.2±1.43	77.9±1.27	+18.9±2.05	0.0	-7.23
19.6±1.49	+2.4±1.71	70.5±2.03	+11.5±2.60	0.14±0.098	-7.09±0.85
23.2±1.53	+6.0±1.74	69.1±1.57	+10.1±2.25	0.15±0.10	-7.10±0.86
21.3±1.69	+4.1±1.89	72.0±1.73	+13.0±2.37	0.0	-7.23
26.4±1.15	—	64.8±1.33	—	0.0	—

Byerly, Knox and Jull (3) have described modifications in the curve for embryonic mortality due to genetic differences in the Beltsville flock, and since they found no significant difference between the second week mortality of embryos from Leghorns and Rhode Island Reds, and since no significant differences attributable to breed exist in the experimental data concerning second week mortality in these experiments, data from Rhode Island Red and Leghorn pens have been summarized together in the present paper.

The diets fed the confined birds used in the experiments summarized in Table I were compounded as follows:

Basal feed mixture:	Parts, by weight
Yellow corn meal (ground yellow corn).....	50
Wheat bran.....	24.5
Oat meal.....	15
Alfalfa leaf meal.....	5.5
Mineral mixture (see text)	
Cod-liver oil.....	2
Protein supplement.....	20

The mineral mixture consisted of such amounts of ground limestone and steamed bone meal as were required to give the desired Ca/P ratio which was from 2:1 to 4:1 in the various experiments; 0.5 part of common salt and 0.5 part of anhydrous sodium sulphate. The basal diet of course was as described except that it contained 20 parts of the basal feed mixture instead of the protein supplement.

Other lots of birds were used to test the value of bluegrass range as a supplement to all-vegetable diets; to test various feedstuffs for toxicity; to test the effects of various Ca/P ratios on second week mortality and incidence of chondrodystrophy and to test the deficiency of individual feedstuffs when fed alone. Material and methods used in these experiments are described later in this paper.

### Mortality Curves

Four typical mortality curves are given in Figure 1 to demonstrate the sharply defined peaks in mortality more clearly than can be done in the tables. Diets described as giving high second-week mortality of embryos all gave the sharp peak, or a more pronounced one, shown for the birds on the basal diet fed in confinement in Figure 1A.

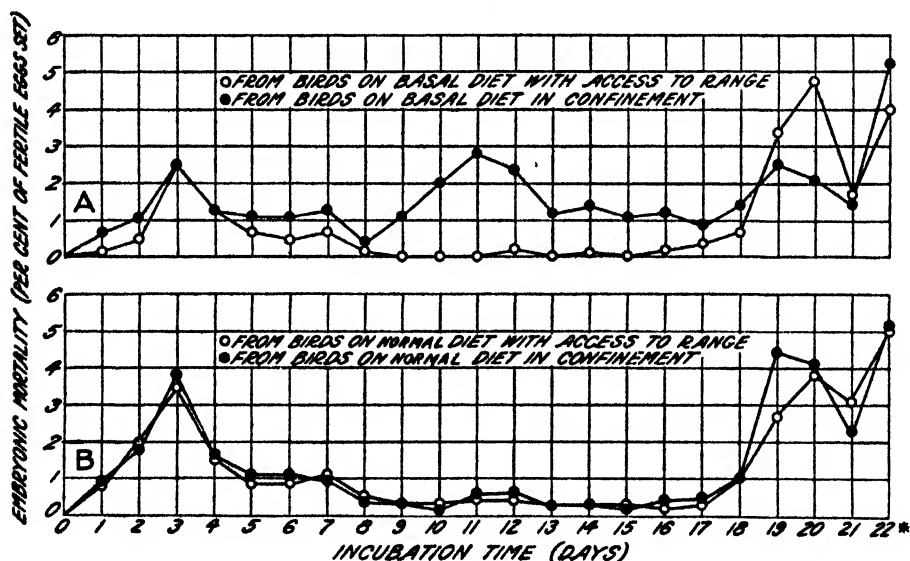


FIG. 1.—Embryonic mortality curve as affected by variations in diet of the parent stock.

A.—○. Mortality of embryos in eggs from birds receiving the basal diet with access to bluegrass range.

●. Mortality of embryos in eggs from birds receiving the basal diet in confinement.

B.—○. Mortality of embryos in eggs from the breeding flocks.

●. Mortality of embryos in eggs from birds receiving the normal diet in confinement.

\* Alive on the 22nd day of incubation but not hatched.

The percentage mortality of fertile eggs on each day of the incubation period is plotted against incubation time in Figure 1B for the breeding flock data. The mortality curve so obtained is very similar to that pub-

lished by Payne (16) for the fowl and by Riddle (18) for the dove. It does not contain a peak during the second week of incubation which Byerly (1) reported as of frequent occurrence. Byerly based his statement at that time on data from both confined and free range birds though the significance of this fact was not then appreciated.

The curve for embryonic mortality in eggs produced by hens receiving a "normal" diet in confinement during the three years of experiment is plotted in Figure 1B as well. There is very close agreement between this curve and the curve for embryonic mortality in embryos from the breeding flocks. This demonstrates the adequacy of the normal diet used for the production of eggs capable of sustaining embryonic life in normal fashion.

The mortality curve for embryos in eggs from confined birds on the basal diet is given in Figure 1A. It will be noted that a relatively large portion of the mortality occurred during the second week of incubation, during which period the mortality was very low in embryos in eggs from the breeding flocks and confined birds on the normal diet. A mortality curve for embryos in eggs from birds on a similar basal diet, to be described later, but with access to range is also plotted in Figure 1A. This curve lacks the high second-week mortality shown in the curve for embryos in eggs from birds fed the basal diet in confinement. This demonstrates that some feed or feeds obtained from the range used are capable of supplying a deficiency existing in the basal diet. The protein concentrate in the normal diet, consisting of 8 parts meat meal, 7 parts North Atlantic fish meal, and 5 parts of dried buttermilk, is shown to satisfy this deficiency by the mortality curve in Figure 1B.

#### *Effects of Protein Concentrates on Embryonic Mortality and Chondrodystrophy*

Table I contains statistical comparisons<sup>1</sup> between the relative embryonic mortality in eggs from hens on several diets expressed as per cent of fertile eggs set. That portion of the data from the breeding flocks which was derived from the Single Comb Rhode Island Red and Single Comb Leghorn flocks of the same breeding as the experimental birds is included for comparison.

The difference in mortality, in hatchability of fertile eggs set, and in percentage of chondrodystrophy among fertile eggs set, in each period, between the normal and each of the other diets, are given with their probable

<sup>1</sup> The probable error of the mean of the values for the hens on each of the diets and the probable error of the differences between those means are the statistical measures of significance used.

TABLE

EMBRYONIC MORTALITY, HATCHABILITY, AND CHONDRODYSSTROPHY

	Diet	Birds	Eggs	First week		Second week	
				Mortality	Difference corn and other diets	Mortality	Difference corn and other diets
		No.	No.	Per cent	Per cent	Per cent	Per cent
Corn	Reared and fed	8	227	23.3±3.56	—	11.6±2.46	—
	Fed	5	332	39.8±10.01	—	13.6±1.31	—
Wheat	Reared and fed	9	534	25.8±2.37	+2.5±4.27	44.2±5.62	+32.6±6.13
	Fed	4	115	96.8±1.48	+57.0±10.1	1.3 ±0.63	-12.3±1.45
Soy bean	Reared and fed	1	18	16.7	-6.6	16.7	+5.1
	Fed	3	90	64.6±5.94	+24.8±11.7	12.7±0.20	-0.9 ±1.33
Butter-milk	Fed	2	32	77.5±0.34	37.7±10.0	0±0.0	-13.6

error. These values indicate the probable effect of diet on each of the factors named. The same method of comparison is used for Tables II, III, and IV with other appropriate diets as the base of comparison in each case.

The mortality during the first week of incubation was significantly greater than that on the normal diet in eggs from birds receiving the basal diet supplemented with soybean meal, with meat meal, and with North Atlantic fish meal; significantly lower first-week mortality was obtained only on the diet containing the yeast preparation. These differences are all relatively small, 58 per cent or less of the value for eggs from hens on the normal diet, whereas differences in second-week mortality are relatively great being about 620 per cent of the normal in case of the eggs from birds receiving soybean meal.

It is evident that the mixture of protein concentrates used in the normal diet, the buttermilk, crab meal, and North Atlantic fish meal have satisfied the deficiency in the basal diet. Eggs from birds receiving diets containing these supplements supported life during the second week of embryonic development as well as did the eggs from birds in the breeding pens which

## IV

## IN EGGS FROM PULLETS RECEIVING A SINGLE SOURCE OF PROTEIN

Third week		Chicks hatched		Chondrodystrophy	
Mortality	Difference corn and other diets	Total	Difference corn and other diets	Incidence	Difference corn and other diets
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
16.0±3.27	—	49.1±3.96	—	2.88±0.87	—
24.8±4.32	—	21.8±5.87	—	0.80±0.95	—
13.8±2.47	-2.2±4.09	16.2±2.47	-32.9±4.66	10.3±1.67	+7.42±1.89
1.2±1.06	-23.6±4.45	0.7±0.63	-21.1±5.90	0.0	-0.80
5.6	-10.4	61.0	+11.9	0.0	-2.88
18.7±3.64	+2.7±4.40	4.0±2.00	-17.8±6.20	0.0	-0.80
12.5±1.01	-3.5±2.48	10.0±0.67	-11.8±5.91	0.0	-0.80

were kept on range and fed a normal diet. The other diets all gave significantly higher second-week mortality and incidence of chondrodystrophy than the normal diet except the diets containing Menhaden fish meal and the yeast preparation. The Menhaden diet was fed to only a small lot of birds; embryonic mortality in the eggs from these birds was significantly higher than normal during the second week of incubation while the incidence of chondrodystrophy was higher but not significantly so, possibly owing to the small number of birds used. The yeast preparation may have had some supplemental value; more data are needed.

The results obtained with meat meal were quite variable. During the first year the diet gave fairly good results, as reported by Byerly, Titus, and Ellis (2) though the incidence of chondrodystrophy was higher than that in eggs from birds receiving other animal protein concentrates during the same year. During the 1930-31 experiment, the pens receiving meat meal gave poor results. The difference, in the authors' opinion, is probably due to differences in treatment of the meat meal during the process of manufacture.



It will be noted that ground whole soy beans gave a lower second-week mortality than soybean meal, the difference being  $7.7 \pm 2.22$  per cent, although second-week mortality in eggs from the ground soy bean lot was significantly higher than that in eggs from birds on the normal diet. One lot of birds receiving ground soy beans of a small black variety (Wilson) gave results equal to those obtained from the normal diet. The soybean meal and the other lots of ground soy beans were manufactured from a larger, yellow variety of bean (Mammoth Yellow).

Third week mortality differed significantly from the normal only in the case of eggs from the birds on Menhaden fish meal. This striking uniformity in third-week mortality is only apparent because, of course, in those eggs from groups showing high mortality during the first two weeks, there were relatively fewer live embryos during the third week. The uniformity in percentage mortality during the third week of incubation therefore means that mortality during the third week of incubation was proportional to mortality during the first two weeks. This relatively increased mortality was probably indirectly due to the same cause or causes as the second-week mortality. Second-week mortality seems thus far to be more or less specifically related to the diet of the hen while mortality during the first and third weeks of incubation varies with many environmental factors other than diet.

It has been pointed out by Byerly, Titus, and Ellis (2) that high second-week mortality and chondrodystrophy are associated. It now seems likely from the work of Landauer (11) on the development of an inherited type of chondrodystrophy (the Creeper variety of fowl) that chondrodystrophy is a direct result of retardation of the growth of the embryo early in incubation. It is likely that the high incidence of chondrodystrophy in some of the present experiments only indicates that the deficiency in the eggs was operative at the critical period of growth described by Landauer. Whatever the nature of the deficiency that caused high second-week mortality, the same deficiency probably caused the associated high incidence of chondrodystrophy in these experiments.

Byerly, Titus, and Ellis (2) confirmed the Hutt and Greenwood (8) report that the incidence of chondrodystrophy cannot be attributed to chance. They also showed that the incidence of chondrodystrophy was apparently greater in the progeny of hens receiving feedstuffs of vegetable origin than in those containing an animal protein concentrate. It is apparent from the data in Table I that the birds on diets of vegetable feedstuffs have shown a relatively high incidence of chondrodystrophy

throughout the three years of the experiment. The meat meal diet gave a higher incidence during the subsequent experiments than in the first year although it was at that time noted that the meat meal diet gave the highest incidence of chondrodystrophy of any of the animal protein-containing diets used in that year. Menhaden fish meal seems to fall in the same class with meat meal. It was used only during the second year of the experiment.

#### *Hen Variation and Chondrodystrophy and Embryo Mortality*

In order to test further the effect of diet on incidence of embryo mortality during the second week of incubation and on the incidence of chondrodystrophy, pullets which produced such embryos during the experimental periods of 1929-30 and 1930-31 were segregated in the following experimental period. If there is any inherent tendency of certain birds to produce such embryos, these birds should give more convincing evidence of the role of diet than an unselected group.

The data obtained from these birds are summarized in Table II. It will be noted that the second-week mortality of embryos from hens on the normal diet is not significantly different from that of embryos from unselected pullets on the same diet (Table I), the difference being  $1.7 \pm 0.93$ . The embryos from birds on the basal diet showed a significantly higher second-week mortality than those on the normal diet, the difference being  $13.1 \pm 1.91$ . The incidence of chondrodystrophy was significantly decreased on both the basal and the normal diet from that of the progenies of the same birds in the preceding year. The incidence of chondrodystrophy and of second-week mortality on the basal diet are not significantly higher than those shown by unselected pullets on the same diet. This probably means that the evidence adduced by Hutt and Greenwood (8) and Byerly, Titus, and Ellis (2) that chondrodystrophy is significantly more frequent in the progenies of some birds than others indicates a temporary physiological idiosyncrasy of those birds rather than a constant or inherent tendency toward the production of chondrodystrophic embryos.

The group indicated as cottonseed meal-soybean meal received cottonseed meal for six months of the period, then soybean meal for the remainder. These products were substituted for 40 parts of the basal feed mixture. This lot, though small, confirms the results obtained with these same products with pullets. There was no apparent difference between the data from the two portions of the experimental period so they were combined.

It has been noted that the chondrodystrophy-producing hens which re-

ceived the normal diet, produced eggs which sustained life through the first two weeks of incubation to a degree not significantly different from that of eggs produced by unselected pullets on the same diet. The third week mortality, however, is significantly greater in the eggs from the chondrodystrophy-producing hens. This may be due to the large size of the eggs laid by hens on this diet due to its high protein content of about 21 per cent, or to some other factor impossible to determine from the data at hand.

### *All Plant Source Diets and Range*

Table III contains data from range birds. All birds fed the diets indicated were F<sub>1</sub> pullets from a cross between Jersey Black Giants and Single Comb White Leghorns. Experimental flocks received basal, soybean meal, linseed meal, and peanut meal diets, and in addition there were breeding flocks which received a normal diet. The birds were housed in colony laying houses in lots of 25 pullets. Each house was located in a plot of ground about 900 sq. m. in area covered with weedy blue grass. Cockerels of the same breeding as the pullets were used and were rotated from pen to pen each day among the experimental flocks. The basal diet used for one series of pens was that used for the confined birds. The basal for the duplicate series contained ground wheat and middlings instead of bran, and ground oats instead of oatmeal. Since no statistically significant differences were found between similar pens, they have been summarized together.

These eight pens of birds fed all-plant source diets gave good results. The data indicate that the birds were able to obtain from the range enough of the material lacking in the diet to keep the mortality of their embryos during the second week at a normal figure. The data from birds of the same breeding in the breeding pens show a higher third-week mortality. The latter birds were pen mated and the difference in third-week mortality is probably due to the consanguinity of the males and the females.

### *Non-toxicity of Feedstuffs Permitting High Second-week Mortality and Chondrodystrophy*

In order further to test the properties of feedstuffs known to permit high second-week mortality of embryos, data were gathered from lots of confined birds fed the normal diet supplemented by 20 per cent of wheat bran, rice bran, and alfalfa-leaf meal, respectively. (Rice bran had been found to permit high second-week mortality of embryos when used with diets not discussed in the present paper.) None of these pens gave second-week mortality of embryos significantly different from that obtained in

embryos in eggs from birds on the normal diet. It may be concluded, therefore, that there is no toxic substance present in these feedstuffs that is responsible for this type of embryo mortality.

#### *Variation in Mineral Portion of Diet Without Effect*

Two pens of confined birds, not included in the data in Table I, were fed the normal diet except that the Ca/P ratio was adjusted to 1:1. Second week mortality was not significantly different from that in eggs from birds receiving the normal diet with a Ca/P ratio of 2:1 or 4:1. One pen of confined birds, not included in the data in Table I, received the basal diet with a supplement of 5 parts of dried kelp. The second-week mortality was not significantly different from that in eggs from birds receiving the unsupplemented basal diet.

#### *Certain Protein Sources Permitting Growth and Egg Production are Inadequate for Reproduction*

Data from experiments designed to give evidence as to the degree of the deficiency of some of the constituents of the several diets are given in Table IV. Chicks were reared on a sole diet of corn products, wheat middlings, and ground whole soy beans with dextrin filler, respectively, to find out whether these feedstuffs used alone would give the same or higher second-week mortality and incidence of chondrodystrophy as when used with other feedstuffs as described above. Only one pullet lived to lay eggs in the soy bean lot; so the data from that source are very meagre.

The birds used in this set of experiments were Single Comb White Leghorns. Those reared and fed on wheat middlings received only wheat middlings, cod-liver oil, and a mineral mixture containing calcite, bone meal, salt, and sodium sulphate. They had access to sunlight in a concrete floored yard but had no access either to green feed or the soil. The lot of birds reared and fed on corn, received a mixture of corn meal and corn-gluten meal blended to form a mixture with the same crude protein content as the wheat middlings. This pen received cod-liver oil and mineral mixture, and the breeding and housing were the same as for the middlings pen. Ground soy beans and dextrin, with mineral supplement and cod-liver oil, were used for the soy bean pen. The Ca/P ratio was adjusted to 1.8 to 1 in all three diets.

Corn and whole soy beans are apparently about as deficient as the basal diet, while wheat middlings gave much poorer results, apparently being more deficient than corn or soy beans. Four small supplemental pens of

cross-bred pullets ( $F_1$  from Single Comb Rhode Island Red male  $\times$  White Rock females) well-grown, sexually mature, and vigorous, were placed, one lot of 6 birds on each of the corn, wheat, and soy bean diets, and a fourth lot of 6 birds on a diet of buttermilk, dextrin, mineral mixture, and cod-liver oil. Again the corn diet was better than the wheat middlings diet from the standpoint of embryo mortality. Few of the embryos in eggs from the birds on the wheat middlings diet lived beyond the first three days of incubation. The data from the milk-dextrin diet are so scanty as to demonstrate only the possibility of hatching chicks from eggs produced on such a diet. It should be noted that the few eggs produced on this diet were laid after the birds had received the diet for six months or more.

### DISCUSSION

The data demonstrate clearly that the basal diet used is deficient in some substance or substances necessary for, or at least auxiliary to, embryonic life during the second week of incubation.

This deficiency can hardly be associated with fat or fat-soluble vitamin, for it was eliminated by feeding North Atlantic fish meal, buttermilk, and crab meal, all products of low fat content. Further, all diets used were supplemented with cod-liver oil and all birds had access to sunlight. All diets, except those containing but a single protein source, contained about 50 per cent yellow corn so that vitamin A should not be a limiting factor. Ellis, Miller, Titus, and Byerly (6) have shown adequate vitamin A content of eggs from hens receiving the same cod-liver oil and more deficient diets.

The deficiency cannot be ascribed to improper Ca/P ratio for this was varied from 1:1 to 4.0:1 without correlated changes in second-week mortality or incidence of chondrodystrophy. The same is true for amount of Ca and P for these were varied fairly widely without correlated changes in second-week mortality. The deficiency is probably not satisfied by iodine as present in kelp.

The water soluble vitamins B and G, as at present established, may not be completely ruled out but the evidence is against them. Ellis, Miller, Titus, and Byerly (6) have shown that the vitamin B content of eggs may be varied by diet but that chicks may hatch from vitamin B deficient eggs though they often die of polyneuritis soon afterward. No cases of polyneuritis in chicks hatched from eggs described in the present experiment were observed nor would the constitution of the diets themselves indicate the possibility of vitamin B deficiency.

The experiments of Ellis, Miller, Titus, and Byerly (6) do not indicate pronounced decrease in the vitamin G content of eggs produced on diets very deficient in this factor as compared to the normal diet. On the other hand, some supplements which overcome the deficiency in the basal diet are substances which are fairly rich in vitamin G. This is especially true of the range birds and of those receiving buttermilk. It is possible that vitamin G or some other vitamin plays an indirect role, permitting better assimilation of materials not otherwise utilized.

The qualitative protein content of the diet itself may be the effective factor. Evidence on the possibility of varying the quality of the protein content of eggs is thus far on a somewhat insecure basis. McFarlane, Fulmer, and Jukes (13) were unable to show variation in amino acid distribution due to diet. On the other hand, Pollard and Carr (17) and Gerber and Carr (7) have reported data on pigeon eggs which they interpreted as showing that amino acid distribution in such eggs may be affected by diet and that such changes affect the hatchability of pigeon eggs. Titus, Byerly, and Ellis (19) have reported data which they interpreted as indicating differences in crude protein content of eggs due to diet fed the hens producing them. Calvery and Titus (4) were unable to find differences in the corn and wheat diet eggs described in these experiments as to their amino acid makeup. Much work must be done both as to the physical chemistry and organic chemistry of egg proteins before a final conclusion may be drawn as to the effect of diet.

A considerable mass of data does exist, however, that indicates a possible explanation of the failure of some of the protein concentrates to satisfy the deficiency of the basal diet. The work of Maynard (12) on fish meals indicates clearly that the process of manufacture of Menhaden fish meal by the flame-drying process leads to considerable decrease in the biological value of the protein. Other conditions of manufacture of this product probably also affect it adversely. Ingvaldsen (9) has reported destruction of amino acids in Menhaden fish meal by heat.

This effect of heat probably applies to proteins generally. Morgan (15) showed that cereal proteins are affected even by the heat used in the toasting process.

The meat meals used were dry rendered and so also heated to an unknown and probably variable temperature. It is possible that the process of oil extraction from soy beans and cottonseed also caused deterioration of the proteins and this may account for the difference noted between the effectiveness of whole ground soy beans and soybean meal.

The argument may of course be advanced that the reported biological values of the proteins used do not support an hypothesis that qualitative protein deficiencies are in any way responsible for the high second week mortality of embryos and its associated high incidence of chondrodystrophy. Tests of biological value of proteins are not necessarily applicable to experiments on reproduction. Diets adequate for growth and maintenance are not necessarily adequate for reproduction as pointed out by Mitchell (14). In the present experiments, the birds reared on wheat middlings grew faster and appeared to be in better physical condition than those reared on corn products; yet eggs from the latter were distinctly superior for the maintenance of embryonic life.

The period of incubation during which mortality was specifically increased itself suggests some derangement of protein metabolism, for it is in the first half of the incubation period that protein is metabolized in greatest amounts relative to the amount of living material in the egg. Further, many of the dead and moribund embryos were highly edematous.

Many deficiencies in diet and what has been referred to as deficiency in these experiments may be deficiency only in the sense that some of the experimental animals receiving such diets were unable properly to assimilate necessary materials actually present in the diet. This seems to have been true of the birds which produced chondrodystrophic embryos as previously noted. Further, eggs from birds reared on corn, middlings and soy beans, respectively, were less deficient than eggs from birds given these same feeds only after they were well grown. This must mean that among

TABLE V

VARIATION IN EGG PRODUCTION AND HATCHABILITY OF EGGS PRODUCED BY BIRDS IN CONFINEMENT RECEIVING THE NORMAL AND BASAL DIETS, RESPECTIVELY, FOR ONE ENTIRE ELEVEN-MONTH PERIOD

Diet	Birds	Hatchability	Coefficient of variation	Egg production	Coefficient of variation	Correlation coefficient egg production and hatchability
Basal. ....	No. 164	Per cent 56.8	$48.1 \pm 1.99$	No. 104.7	$32.5 \pm 1.33$	$+0.155 \pm 0.0768$
Normal. ....	151	66.4	$27.2 \pm 1.13$	148.1	$25.8 \pm 1.06$	$+0.136 \pm 0.0803$
Difference. ....	—	—	$20.9 \pm 2.29$	—	$6.7 \pm 1.71$	—

the birds *reared* on these feeds, those birds less able to utilize them died before reaching maturity.

In Table V data are tabulated from birds which survived an entire experimental period on the normal and on the basal diet, respectively. The coefficients of variability for the hatchability of eggs and for the number of eggs produced are higher for the birds on the basal diet. This is the expected result since increased variability in experimental animals is in itself often considered an evidence of inadequate diet.

Correlation between egg production and hatchability is positive but not significant for the birds on each diet. This means that the birds varied in the efficiency with which they transferred materials necessary for embryonic growth from the diet to the egg. The birds which laid few eggs were not able to store materials present in minimal quantities and thus to produce better eggs; if anything, those that were efficient, able to convert larger amounts of feed into eggs, even though the feed supplied was deficient, were also able to produce eggs more capable of supporting embryonic life. This is a very important matter from the practical standpoint for the opinion is popularly held that the barnyard fowl, which produces few eggs, must consequently produce better eggs from the standpoint of human nutrition than the bird bred and fed to lay many eggs. If the ability of an egg to sustain embryonic life is an index of its value as human food then the eggs from the efficiently managed high-producing flocks are at least as desirable as any others.

It is recognized, of course, that treatment in the process of manufacture, especially with heat, may reduce the vitamin potency of the protein concentrates used. In the case of the vitamin G potency of meat meal this must be true for, whereas lean meat is supposedly a fair source of vitamin G, the experiments of Ellis et al., show that the meat meal used in these experiments was very deficient.

### CONCLUSIONS

Diets consisting of certain cereal products supplemented with alfalfa-leaf meal, minerals, and cod-liver oil caused the production of eggs the embryos in which showed a significantly greater mortality during the second week of incubation than the embryos in eggs from birds on a diet supplemented with certain protein concentrates of animal origin.

It is concluded that corn, wheat, ground soy beans, soybean meal, cottonseed meal, flame-dried Menhaden fish meal and some lots of meat meal are deficient in some substance necessary for the production of eggs



capable of supporting embryonic life during the second week of incubation.

It seems likely that factors in the process of manufacture of the deficient protein concentrates, especially excessive heat, may be responsible for their deficiency.

Vacuum dried North Atlantic fish meal, steam-dried crab meal, buttermilk, a combination of buttermilk, North Atlantic fish meal, and meat meal, and free range were found to enable the production of eggs capable of supporting embryonic life through the second week of incubation in normal fashion.

Evidence as to the nature of the deficiency is somewhat conflicting. The proteins in the deficient concentrates may well have been qualitatively deficient. It is possible that vitamin G or some one of the less well known water soluble vitamins is concerned although the authors have thus far been unable to obtain experimental evidence for such a view.

There was no tendency for the birds that laid fewer eggs when receiving deficient diets to lay eggs more capable of sustaining embryonic life than those laid by birds laying a greater number while receiving the same deficient diet.

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## EFFECT OF DIET ON EGG COMPOSITION

### III. THE RELATION OF DIET TO THE VITAMIN B AND THE VITAMIN G CONTENT OF EGGS, TOGETHER WITH OBSERVATIONS ON THE VITAMIN A CONTENT

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THIS paper presents a series of vitamin investigations which have formed a part of a study on the effect of diet on the production, size, composition, vitamin content, nutritive value, and hatchability of eggs. Papers on the chemical composition of the eggs and on the embryonic mortality have already appeared (1, 2, 3). The present studies have been directed at the influence of the diet on the content of the egg in the factors associated in the vitamin B complex. Particular attention has been given to the vitamin B and vitamin G content of eggs produced on diets which showed a considerable variation in these vitamin factors and in hatchability. Because of a wide difference in pigmentation of the eggs produced on the different diets, all of which contained cod-liver oil, the vitamin A content was determined on a normally pigmented lot of eggs in comparison with another lot nearly devoid of yellow pigments.

Recent investigations (4) have confirmed earlier reports that vitamin G ( $B_2$ ) as well as vitamin B ( $B_1$ ) is essential for the growth of the chick while the  $B_3$  factor apparently is not essential.

While the vitamin content of eggs, like that of milk, possibly is not affected by external factors to the extent that plant materials may be affected (5), alterations in the supply of vitamin A and of vitamin D in the diet of the hen have been found to influence the composition of the egg (6). Likewise direct irradiation of hens may improve the hatchability of the eggs (7, 8).

In the usual case the work on the vitamin B complex and on vitamins B and G has been directed toward the relative richness of eggs in the sev-

eral factors rather than toward the effect of the diet of the hen on the eggs. Steenbock (9) prepared a water-acetone-soluble fraction from egg yolk, small doses of which cured pigeons suffering from polyneuritis. Osborne and Mendel (10) in feeding tests with rats showed that eggs were approximately three times as potent in vitamin B (complex) as milk. Hoagland and Lee (11) used pigeons to determine the amount of the antineuritic factor in eggs. They found that a diet of polished rice and dried eggs did not fully protect against polyneuritis even when the eggs were fed at a 30 per cent level. They cited the work of Cooper (12) as confirming their results which indicated that eggs are relatively low in the antineuritic vitamin. Recent work (13) has shown that egg white is devoid of vitamin B or the antineuritic factor but is rich in vitamin G ( $B_2$ ), while egg yolk contains both factors (14).

As the result of work on the nutrition of tissue cultures, Heaton (15) concluded that factors associated with or identical with vitamins B and G are concerned in the multiplication of tissue cells. Not only was a thermolabile substance found necessary but a thermostable substance as well for the propagation of cells of chick embryo tissue. There was a difference in the response of different tissues. In addition, an inhibitory substance was found in yeast. These findings suggest that additional factors of a dietary nature may be concerned in hatchability.

#### PLAN OF STUDY

The general plans of the hen feeding experiments, together with results on hatchability and chemical composition, have been discussed in other papers (1, 2, 3). The vitamin studies on eggs have dealt with the effects on the eggs of a number of diets fed to hens under like environmental conditions. The birds were confined in long laying houses subdivided into pens, each of which was connected with a sunlit concrete floored runway. The work has extended over three years, in each of which new lots of birds were placed on experiment in September and fed until the following August.

The hen diets which have been compared fall into two groups.<sup>1</sup> The diets

<sup>1</sup> The diets used during the first year's work have been described by Titus, Byerly, and Ellis (2). Except for minor changes in the proportions of certain constituents, particularly as they affected the Ca:P ratio, and in diet numbers all the hen diets used in the present experiments are identical to or modifications of those just mentioned. Thus, the basal diet of Group A corresponds to diet 9, while the normal diet which contained the animal products corresponds to diet 8. Likewise, the basal diet of Group B corresponds to diet 10, the yeast 1 (B3) diet to diet 11, and the autoclaved yeast (B4) diet to diet 12. The other diets (A2, A3, B1, B2 and B3 with yeast 2) were introduced in the second or third year of the experiment.

in Group A contained a basal mixture of yellow corn, 500 parts; wheat bran, 245 parts; oatmeal, 150 parts; and alfalfa leaf meal, 55 parts; while those in Group B contained pearled hominy and desiccated meat meal in such proportion as to give 20 per cent protein in the mixture. The basal mixture in the first case was relatively rich in the vitamin B-complex, while in the second it was decidedly deficient. All diets were fortified with respect to vitamin A, vitamin D, and mineral deficiencies by the addition of 2 per cent of cod-liver oil and appropriate mineral substances.

In addition to the basal diets for each group, other diets were studied in which a portion of the basal mixture was replaced by supplements, either of a single feedstuff or of a combination of feeds. The list of diets was as follows:

Group A diets with a vitamin adequate basal mixture

Diet A—the basal mixture without supplement.

Diet A1—the normal diet. Contains 7 parts of fish meal, 8 parts of meat meal, and 5 parts of dried buttermilk to 80 parts of Diet A.

Diet A2—20 parts of rice bran to 100 parts of Diet A1.

Diet A3—20 parts of dried whey to 80 parts of Diet A.

Group B diets with the vitamin deficient hominy-meat meal base

Diet B—the deficient basal mixture.

Diet B1—15 parts of rice polish to 85 parts of Diet B.

Diet B2—15 parts of rice bran to 85 parts of Diet B.

Diet B3—15 parts of dry yeast to 85 parts of Diet B.

Diet B4—15 parts of autoclaved dry yeast to 85 parts of Diet B.

Dry yeast from two manufacturers was used in successive years and has been designated as yeast 1 and yeast 2. Yeast 1 was autoclaved for 4 hours at 15 pounds pressure for use in diet B4.

Fresh eggs produced during the period of maximum production, from February to July, were used in the rat feeding experiments. The rats were confined in individual cages equipped with 3-mesh screen bottoms. In the vitamin B and vitamin G experiments, the young rats were placed on the deficient diet at the age of 4 weeks. When further gains in weight had ceased after 7 to 14 days, therapeutic feeding of eggs and hen diets was initiated. These materials were fed on a 6 day per week basis for an 8 week period, at levels estimated to produce an average weekly gain of approximately 4 grams.

The vitamin A tests were made by the usual therapeutic method. The young rats were depleted of their vitamin A stores (as evidenced by cessation of growth) in 5 to 6 weeks. The eggs were fed 6 days per week for 8

weeks. Weekly weight records on gains in weight and food consumption were kept.

The studies on eggs were divided into 6 series as follows:

1. The growth response of rats to a test dose of whole egg produced on the normal diet and fed at the same level with different basal rat diets which varied in the presence and source of the vitamin B complex and the separate factors or groups of factors designated as vitamins B and G.
2. The distribution of the factors in the vitamin B complex in the yolks and whites of normal eggs.
3. The influence of hen diets from Groups A and B on the vitamin B complex in the whole egg.
4. The relationships between the vitamin B content of hen diets from Groups A and B and of the eggs produced by the hens.
5. The relationships between the vitamin G content of hen diets from Groups A and B and of the eggs produced.
6. The relative constancy and high potency of eggs in vitamin A produced on the basal diets of Groups A and B, supplemented with cod-liver oil but variable in the vitamin B complex and in pigmentation.

#### THE EXPERIMENTS

**Series 1.**—Eggs produced by hens on the normal diet (A1) were fed in test doses to measure the response of the rats on different test rations. The chief purpose of this study was the evaluation of methods for estimation of the vitamin B and vitamin G content of eggs, together with the possible detection of additional factors present in eggs.

Three basal rat diets used with or without modifications in the series of 7 rations were as follows:

Diet 147—Dried egg white, 20 per cent; dextrinized rice starch, 65 per cent; mineral mixture (16), 4 per cent; agar, 1 per cent; and lard, 10 per cent. Cod-liver oil was fed separately.

Diet 149—Like diet 147 except purified casein replaced the egg white.

Diet 191—Purified casein, 18 per cent; dextrinized rice starch, 48 per cent; salt mixture (16), 4 per cent; lard, 8 per cent; cod-liver oil, 2 per cent; and ground white corn, 20 per cent.

The seven comparisons fall into a number of groups according to vitamin deficiency. The vitamin B complex was measured in one case (I) where diet 149 was fed alone. Three diets contained sources of vitamin G in the form of (II) alkaline autoclaved yeast supplied at a 0.4 gram per day level, (III) egg white contained in diet 147, and (IV) neutral auto-

claved yeast supplied at a 0.3 gram per day level. All these diets were deficient in vitamin B, with possible differences in the supply of additional factors. Two other diets contained sources of vitamin B but were deficient in vitamin G. Rice polish extract (V) at a level which protected against polyneuritis but permitted only maintenance of weight was used in one case, and white corn (VI) contained in diet 191 in the other. In the last case (VII) rice polish extract as used in (V) and alkaline autoclaved yeast as used in (II) were both added to diet 149. Possibly this combination still left a deficiency in one or more of the newer factors associated with the vitamin B complex (17).

Averaged results on control rats on the several diets and dietary supplements are shown in Figure 1. The two autoclaved yeast preparations caused little, if any, difference in the response of the rats. The "alkaline" product was autoclaved at pH 9 for 2 hours while the neutral or untreated product was autoclaved for 4 hours. The pressure was 15 pounds in each case.

Rice polish (18) compared favorably with the extract of rice polish as judged by relative richness in vitamin B and lack of vitamin G. The extract was the concentrate from an extraction of rice polish with 85 per cent alcohol. Numerous tests have shown that the extract is a relatively pure source of the vitamin B fraction. Its addition to diet 149 at a level equivalent to 0.5 gram of rice polish permitted little more than maintenance of weight over extended periods of four to five months. During the third month, the rats developed the symptoms ascribed to vitamin G deficiency (17). The most noticeable effects were the skin disturbances, loss of hair, and general emaciation. The addition of autoclaved yeast relieved this condition and promoted a moderate rate of gain.

In keeping with the findings of Chick and Roscoe (13) egg white was found an excellent source of vitamin G with little contamination of vitamin B. A supplement of autoclaved yeast was without benefit but rice polish and wheat germ were effective (Figure 1) in the prevention of polyneuritis and the promotion of growth. No harmful effects such as those noted by Parsons (19) on high levels of egg white were observed on the level which was fed.

The use of white corn as a source of vitamin B (diet 191) was unsatisfactory owing to the irregularities in the behavior of the control rats. Certain ones showed the desired evidence of deficiency in vitamin G but others showed a rather abrupt change after approximately five weeks. From this point they gained slowly without interruption. This behavior may have been due in part at least to coprophagy.

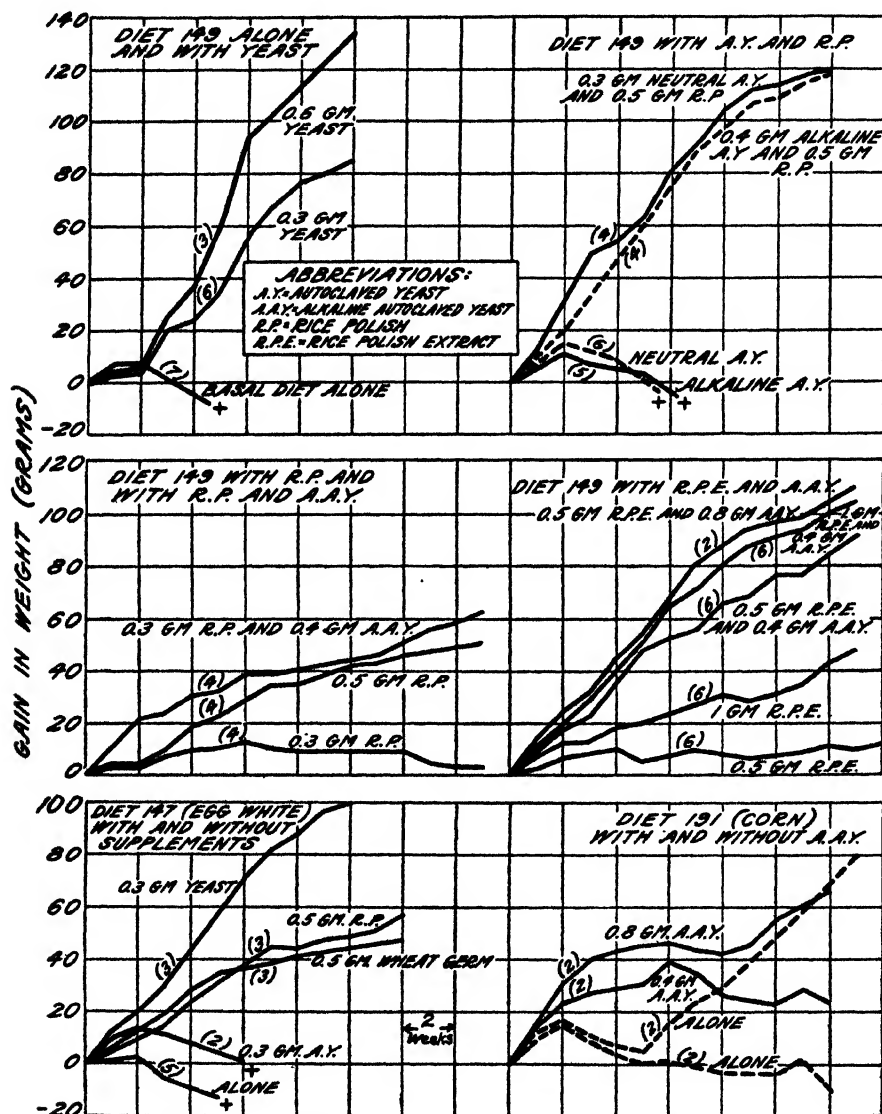


FIG. 1.—Average gains or losses in weight of 97 rats used as controls in the experiments on the vitamin B-complex, the vitamin B and the vitamin G content of hen diets and eggs. The numbers in ( ) refer to the number of rats on which the averages are based. The average time of death is indicated by the symbol +. The supplemental feedings are all given on a daily basis.

Following a 10 to 14 day depletion period, groups of 6 rats evenly divided as to sex and distributed among the 7 dietary treatments so there was never more than 1 rat from a litter on a particular treatment were fed 2 cc. doses of the whole mixed egg daily for an 8 week period. The average results are shown in Table I. The lowest gain was made by Group I on the

TABLE I

RESULTS OF COMPARISON OF METHODS OF DETERMINING THE FACTORS IN THE VITAMIN B COMPLEX WITH A DAILY DOSE OF 2 CC. OF EGGS PRODUCED ON A NORMAL DIET

Rat diet and supplements fed	Ration deficient in	Number of rats	Initial weight on experiment	Eight week test period			
				Initial weight on egg	Total gain	Weekly gain	Daily feed
(I) Diet 149 only . . . . .	Complex	6	gm. 49	gm. 53	gm. 23	gm. 2.9	gm. 3.7
(II) Diet 149 with alkaline autoclaved yeast . . . . .	B	6	47	65	34	4.3	4.1
(III) Diet 147 containing egg white . . . . .	B	6	47	61	32	4.0	3.6
(IV) Diet 149 with neutral autoclaved yeast . . . . .	B	6	46	59	44	5.5	4.1
(V) Diet 149 with extract of rice polish . . . . .	G	6	49	60	42	5.3	4.9
(VI) Diet 191 containing white corn . . . . .	G	6	46	61	94	11.8	8.7
(VII) Diet 149 with sources of B and G	Neither B nor G	6	46	64	97	12.1	7.2

diet containing no vitamin B complex. Of the 3 groups on diets designed to measure the vitamin B content of the eggs, the one on egg white made the least and the one on neutral autoclaved yeast the greatest gain. The average gain of the rats on the neutral autoclaved yeast ration was 44 grams as compared to 32 and 34 grams respectively on the egg white and alkaline autoclaved yeast rations. The alkaline autoclaved yeast has appeared to be the most satisfactory supplement from the standpoint of the constancy of the product. It has been used throughout the work as a vitamin G supplement in determinations of the comparative vitamin B contents of the eggs produced on the various diets.

The two groups on vitamin G tests showed widely different behavior.



The behavior of control animals on diet 191 has been noted. In view of the growth attained by the controls, it was not surprising to find that 2 cc. of egg enabled the group of 6 rats to gain 94 grams in the 8 week period. This was comparable to that attained by the group (VII) which received both the autoclaved yeast and rice polish extract supplements. The 6 rats which received diet 149 plus the rice polish extract and 2 cc. of whole egg (Table I) gained an average of 42 grams in 8 weeks or slightly over 5 grams per week. The performance on this ration has appeared to warrant its use as a measure of the vitamin G content of the eggs.

*Series 2.*—Feeding tests on egg yolks from eggs produced on normal mixed diets were made with rat diets 147 and 149. The results of these tests are given in Table II. Twelve rats arranged in 2 groups received 1 cc. of yolk daily for 6 weeks, then for 4 weeks one group received a 0.5 gram daily supplement of rice polish rich in vitamin B and the other group 0.3 gram of autoclaved yeast as a source of vitamin G. The 1 cc. dose of egg yolk permitted an average weekly gain in weight of 1.9 grams during the first 6 weeks of the test. Rice polish did not materially increase the growth rate in the second period of 4 weeks, while autoclaved yeast did cause an increase from 1.5 to 5.0 grams per week. Thus, vitamin G appeared to be the limiting factor in the yolks.

Three groups of 3 animals each, shown in Table II, which received diet 149 with a daily dose of 2 cc. of yolk gained an average of 7.2 grams per week during an 8 week period. In a subsequent 2 week period, one group received the neutral autoclaved yeast, another the alkaline autoclaved yeast, and the third no additional supplement. Since the rats had passed through the cycle of most rapid growth by the time this period was reached, the weekly averages were low for all 3 groups. The autoclaved yeasts did not promote an increased rate of growth over the control group. However, the results again suggest that the neutral autoclaved yeast retained more of the vitamin B complex than the alkaline treated yeast.

Six rats received diet 147 which contained egg white as the source of vitamin G as well as protein. One group of 3 rats received 1 cc. and the second group of 3 received 2 cc. of egg yolk daily for 7 weeks, then for 3 weeks one rat in each group received rice polish, another neutral autoclaved yeast and the third alkaline autoclaved yeast. The average weekly gains for the 7 week period were slightly better than those on diet 149. The response to rice polish supplement during the subsequent 3 week period showed that vitamin B was the limiting factor on diet 147. Egg white, as shown by the results on diet 147 as well as from tests on the liquid

TABLE II  
THE RELATIVE VITAMIN B AND VITAMIN C CONTENT OF EGG YOLK AS SHOWN BY THE GROWTH RESPONSE OF RATS ON BASAL DIETS WITH AND WITHOUT THE ADDITION OF SUPPLEMENTS RICH IN THESE VITAMINS

Feeding	Period I				Period II			
	Duration	Number of rats	Average		Supplement	Duration	Average	
			Weekly gain	Daily feed			Weekly gain	Daily feed
Diet 149 with 1 cc. of egg yolk (Average)	days		gm.	gm.		days	gm.	gm.
	42	6	2.3	5.2	.5 gm. rice polish	28	2.8	4.2
	42	6	1.5	5.5	.3 gm. aut. yeast	28	5.0	5.1
		—	1.9	5.4				
Diet 149 with 2 cc. of yolk (Average)	56	3	7.8	6.0	No addition	14	1.2	5.5
	56	3	7.3		.3 gm. aut. yeast	14	3.0	5.2
	56	3	6.5	—	.4 gm. alk. aut. yeast	14	2.0	5.5
		—	7.2					
Diet 147 with 1 cc. of yolk (Average)	49	1	4.3	5.3	.5 gm. rice polish	21	18.3	9.1
	49	1	3.0	4.9	.3 gm. aut. yeast		3.7	3.7
	49	1	1.3	5.3	.4 gm. alk. aut. yeast		1.0	3.8
		—	2.9	5.0				
Diet 147 with 2 cc. of yolk (Average)	49	1	5.7	5.5	.5 gm. rice polish	21	13.0	7.2
	49	1	8.7	6.4	.3 gm. aut. yeast		8.3	6.0
	49	1	9.0	5.3	.4 gm. alk. aut. yeast		2.7	5.8
		—	7.8	5.7				

TABLE III  
THE VITAMIN B-COMPLEX IN WHOLE EGGS PRODUCED ON DIFFERENT DIETS, TOGETHER WITH THE RESPONSE OF THE RATS TO THE ADDITION OF VITAMIN B AND VITAMIN G-RICH SUPPLEMENTS. VALUES AS AVERAGE GAIN IN GRAMS PER WEEK

Feeding period	Dose of egg	Supplement to Diet 149	No. of rats	Group A: basal hen diet		Group B: deficient hen diet		
				A	A1 normal	B	B3 yeast 1	B4 aut. yeast 1
First 4 weeks (a)*	2	None	4	gm. 7.5	gm. 4	gm. 4.5	gm. 1	gm. -1
First 4 weeks (b)	8	None	4					
Second 4 weeks (a)	2	None	4	4.5	3			
Second 4 weeks (b)	8	None	4			8.3	12.7	11
Third 4 weeks (1a)	2	Rice polish	2	13.5	13.3			
Third 4 weeks (1b)	8	Rice polish	2			11.7	10.5	12.5
Third 4 weeks (2a)	2	Aut. yeast	2	9.0	6.3			
Third 4 weeks (2b)	8	Aut. yeast	2			8.5	15	10
First 4 weeks	4	None	4			2.3	3.7	1.8
Second 4 weeks	4	None	4			2.7	4.0	4.5
Third 4 weeks (1)	4	Rice polish	2			15.5	15.5	15.8
Third 4 weeks (2)	4	Aut. yeast	2			13	5.5	8.7

\* Designations of groups of rats carried through the successive four week periods. The numbers 1 and 2 indicate division of "letter" group in the third period.

material, showed little or no vitamin B but moderate amounts of vitamin G. while the yolk appeared to be the richer in vitamin B.

*Series 3.*—Whole eggs produced on different diets were compared for the content of the vitamin B complex. The hen diets in the comparison consisted of the basal and the normal (A1) of Group A and the deficient mixture, the dry yeast 1 (B3) and the autoclaved dry yeast 1 (B4) of Group B. The eggs were fed daily in the liquid state to groups of 4 rats each. The experimental feeding was divided into 3 periods of 4 weeks each. In the third or final period the rats were paired within each group on added daily supplements of 0.3 gram of rice polish and 0.4 gram of alkaline autoclaved yeast.

The average weekly gains of the rats by 4 week periods on several doses of eggs from the hen diets are given in Table III. The 2 cc. dose of egg from the basal diet and the normal diet of Group A was sufficient for moderate growth. The former diet appeared to have produced eggs of the greater potency in the vitamin B complex. The hens on the deficient diet with the hominy-meat meal base produced eggs which were less potent than those from the adequate diets. Of the 2 groups on 4 and 8 cc. of eggs respectively, the latter produced gains in the rats approximately equal to 2 cc. of egg from the Group A diets. The grade of yeast added at a 15 per cent level to the deficient diet was clearly ineffective in increasing the vitamin B complex in the eggs. The groups on the 2 cc. dose of both the untreated and the autoclaved yeast failed to gain during the first 4 weeks of the test. An increase to 8 cc. in the second period which was continued through the third period permitted normal gains. The 4 cc. dose of these eggs was approximately equal to the 2 cc. dose of eggs from the normal diet. Subsequent tests on this grade of yeast showed that it possessed perhaps only 25 per cent of the potency of the yeast used for the rat feeding tests. The addition of rice polish and of autoclaved yeast in the third period showed that vitamin B was probably the limiting factor in whole eggs, but the supplements did not indicate any undue disproportion between vitamin B and vitamin G due to diet. However, the number of animals was too small to give a definite answer on this point.

*Series 4.*—The vitamin B content of a number of hen diets and of eggs produced on these diets was determined by rat feeding tests in Series 4. The rats were fed a vitamin B free diet which consisted of diet 149 supplemented with a 0.4 gram daily supplement of alkaline autoclaved yeast. The hen diets and the liquid eggs were then fed in test doses for an 8 week period.

TABLE IV  
THE VITAMIN B CONTENT OF THE DIETS FED TO THE HENS AND OF THE WHOLE EGGS PRODUCED ON THESE DIETS AS MEASURED BY THE GAINS OF RATS ON A VITAMIN B-DEFICIENT RATION\*

	Group A: basal hen diet				Group B: deficient hen diet				
	A	A1 normal	A2 rice bran	B	B1 rice polish	B2 rice bran	B3		B4 aut yeast 1
							yeast 1	yeast 2	
Tests on hen diets:									
0.5 gm.† No. rats	3	8	3		4	4			
Gain‡ gms.	0	2.9	5.5		1.9	—, 7			
1.0 gm: No. rat		4			4	10			
Gains, gms.		4.1			4.2	2.8			
5.0 gms: No. rat				6 died			5 died	4 died	4 died
Gain, gms.									
Tests on eggs:									
1 cc.†: No. rats	7	7	7			6			
Gain, gms.	2.3	2.2	3.9			4.0			
2 cc: No. rats	6	17		4	6	6	4	7	4
Gain, gms.	7.3	5.3		2 died	5.9	7.3	died	3.9	2 died
3 cc: No. rats				4			4‡	6	4
Gain, gms.				3 died			2.6	6.5	4.9
4 cc: No. rats				16			6		6‡
Gain, gms.				5.5			3.4		3.0

\* Rats on the vitamin B deficient ration die in 3 to 5 weeks.

† The daily dose fed to rats.

‡ The item of "Gain, gms." gives the average weekly gain for the 8 week test period.

§ One died, average on remainder.

The results of tests on the hen diets are given in Table IV. The table shows the characteristic components of the hen diets, the daily dose to the rat, and the number and the average weekly gains of the rats. The adequate diet of Group A, with or without special supplements, was fully 10 times as potent in vitamin B as the deficient mixture of Group B. Five grams was the maximum dosage of this diet which the rats consumed. The diets supplemented with 15 per cent of yeast also failed to protect the animals, although that designated as yeast 2 showed greater potency than yeast 1. Both rice polish and rice bran, incorporated as 15 per cent of the diet, were effective vitamin B supplements as evidenced by the approximate equality between the normal diet on the one hand and the deficient mixture supplemented with the rice by-products named.

The vitamin B tests on eggs from three years of experiments have been summarized in Table IV. Only the average weekly gains of the groups of rats on various doses of whole eggs are given. A total of 127 rats, exclusive of controls, have been fed eggs produced on 9 diets at levels ranging from 1 cc. to 4 cc. daily for 8 weeks. The normal diet (A1) and the deficient hominy-meat meal diet (the basal of Group B) have received the greatest attention. Seventeen rats on a 2 cc. dose of the former and 16 rats on a 4 cc. egg dose of the latter produced approximately equal growth.

Although relatively small lots of rats ranging in most cases from 4 to 7 were used in the other comparisons, two and sometimes three levels of egg were fed in the usual case. Thus, the effect of a particular hen diet or kind of feed can be measured by the results as a whole from several lots of rats.

The eggs from the normal diet were possibly less potent than from the Group A basal diet without the animal proteins or the normal diet with 16.7 per cent of rice bran. One cc. of egg from the last named diet was sufficient to produce an average gain of 3.9 grams per week. Eggs from hens on the Group A basal diet, fed at 1 cc. and 2 cc. levels, produced gains of 2.3 and 7.3 grams per week respectively, as compared to 2.2 and 5.3 grams on 1 cc. and 2 cc. of eggs from the normal diet. The ability of rice bran and rice polish to raise the vitamin B content of the eggs was also shown when these feeds were added to the deficient mixture in Group B. Yeast 1 failed to materially increase the potency of the eggs although it did furnish some protection against polyneuritis in the hens. The autoclaved yeast did not alter the general order of results. Yeast 2 was reasonably satisfactory as a supplement although not as good as rice bran.

*Series 5.*—This series of tests on the vitamin G content of hen diets and of eggs was analogous in the plan of work to Series 4. The list of diets

differed in the omission of the autoclaved yeast diet and the substitution of the dried whey diet (A3) for the basal diet of Group A. The vitamin G deficient ration fed to the rats consisted of diet 149 with the addition of sufficient extract of rice polish to protect against polyneuritis and permit maintenance of weight.

The data on the vitamin G tests of the hen diets are summarized in Table V. The 0.5 gram dose of the diets based on the vitamin-adequate

TABLE V

THE VITAMIN G CONTENT OF DIETS FED TO HENS AND OF THE EGGS PRODUCED ON THESE DIETS AS MEASURED BY THE GAINS OF RATS ON A VITAMIN G-DEFICIENT RATION\*

Item	Group A—basal hen diet			Group B—basal hen diet			
	A1 normal	A2 rice bran	A3 dried whey	B1 basal	B2 rice bran	B3	
						yeast 1	yeast 2
Tests on hen diets							
Daily dose, gms. . . .	0.5	0.5	0.5	5.0	1.0		2.0
Number of rats† . . .	6	6	5	5	5		5
Weekly gain, gms. . .	3.6	4.3	4.9	1.9	4.1		5.0
Tests on eggs							
Daily dose, cc. . . . .	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Number of rats‡ . . .	12	6	6	12	12	12	6
Weekly gain, gms. . .	4.9	7.9	6.5	4.1	4.5	3.8	6.0

\* Rats on the vitamin G deficient ration alone gained 0.9 grams per week.

† Female rats.

‡ Males and females equally distributed.

mixture was sufficient in all cases to promote weekly gains of 3 to 5 grams. The diet containing 20 per cent of dried whey in place of the combined animal product supplement of the normal diet produced the best gains for the Group A diets. A ten-fold increase in dose was required of the basal diet of Group B for a gain of approximately 2 grams per week. The inclusion of 15 per cent of rice bran or yeast 2 in diets with the deficient mixture of Group B as the base, raised the vitamin G content to a level which made 1 gram of diet of the first and 2 grams of the second named supplement approximately equal to 0.5 gram of the Group A diets. The differences in the diets in vitamin G content were possibly not as pronounced as in vitamin B. However, the 5 gram dose of the deficient diet in Group B approximated a full feed for the rats since they consumed only 0.8 gram of diet 149 per day in addition to the 5 gram dose. Yeast 2 appeared relatively

richer and the rice bran relatively poorer in vitamin G than in vitamin B when fed as components of the hen diets.

All determinations on the vitamin G content of the eggs were made with a 2 cc. dose of egg. Comparisons were made of 5 diets in one year and of 6 diets in another year. Three diets were the same for both years while in a fourth diet yeast 2 replaced yeast 1. Results on the repeated diets showed differences which ranged from 0.1 to 0.4 gram in average weekly gains of the groups of rats. Accordingly, the groups from both years have been combined. The results are included in Table V.

The normal diet, the deficient diet alone and with yeast 1 or rice bran supplement apparently produced no marked differences in the vitamin G content of the eggs. The average weekly gains of 12 rats in each case were 4.9, 4.1, 3.8, and 4.5 grams respectively. Six rats on eggs from the rice polish diet (not included in the Table) gained equally well. With the wide difference noted in the vitamin G content of the hen diets as well as in the vitamin B content of the eggs, the lack of difference in the vitamin G content of the eggs from the diets named is noteworthy. The use of dried whey in place of the combination of animal protein supplements in the normal diet and the addition of rice bran to the normal diet resulted in an increased gain in the rats on egg tests. Paired comparisons were possible in a number of cases where rats of the same litter and sex were used on different eggs. All 6 rats on eggs produced on the rice bran-normal diet gained more than their mates on the same diet without the rice bran and on the deficient diet. Dried whey was no different from the mixed animal protein supplement in its effect on the eggs. In 5 of 6 cases the rats on eggs from the yeast 2 diet gained more than their mates on the deficient diet.

The results as a whole suggest that the hen diets did not affect the vitamin G content of the eggs to the extent which has been noted for vitamin B. Lack of vitamin G in the diet apparently did not result in any pronounced drop in the vitamin content of the egg. On the other hand, the inclusion of foods rich in vitamin G in the normal diet used in these experiments tends to enhance the vitamin G content of eggs.

#### *The Vitamin A Content of the Eggs*

Comparisons of the vitamin A content of eggs produced on the deficient diet with that of eggs produced on the normal diet were made in 1930 and again in 1931. These eggs were chosen because of their wide difference in pigmentation as well as other differences due to diet. The hominy-meat meal diet produced eggs with yolks nearly devoid of yellow pigments,



while the normal diet produced characteristic highly pigmented yolks. Rats which had become depleted of their vitamin A stores as manifested by cessation of growth were given daily feedings of yolk in the 1930 tests. Doses of 0.2 and 0.4 cc. were fed. The averaged gains in weight of the groups of rats during the 8 week period totaled 60 and 62 grams respectively on 0.2 cc. of egg yolk from the deficient and the normal diet. In 1931, the yolk and white were mixed and fed at a 0.1 cc. level. Twelve rats which had gained an average of 80 grams during a depletion period ranging from 37 to 40 days were paired according to sex (3 pairs of each sex), litter and weight. One of each pair was fed daily 0.1 cc. of egg from the deficient diet and the other an equal amount of egg from the normal diet. Numerous cases of xerophthalmia were cured during the early part of the period and at the close of the 8 week test period all animals appeared normal in health. The average gain was 47 grams for the deficient and 32 grams for the normal group. Considered by pairs, the rats in the former group gained more in every case than their mates in the latter group. These results indicate that the diet deficient in vitamin B complex produced eggs with a greater vitamin A potency although greatly lessened pigmentation.

The ability of the hens on the former diet, deficient in vitamin B complex, to store more vitamin A in the eggs than those on normal diet was probably due to a difference in the rate of egg production. The 2 per cent of cod-liver oil which was added to both diets was adequate for storage of a generous supply of vitamin A regardless of the amount naturally contained in the other foodstuffs of the diets.

From the data of Murphy and Jones (20), as well as those of the present study, it appears that 0.1 cc. was more than sufficient to supply 1 unit of vitamin A. On this basis, a 50 gram egg, exclusive of shell, produced on either of the diets studied, supplied over 500 units of vitamin A.

The importance of these results as they relate to hatchability and other factors concerned in the present problem rests in the fact that a simple diet of hominy, meat scrap, minerals, and cod-liver oil produced eggs which were nearly colorless yet adequately supplied with vitamin A. Therefore, vitamin A and probably vitamin D were not concerned in the low hatchability of eggs on the deficient diets studied.

### DISCUSSION

In view of the apparent multiplicity of factors in the vitamin B complex, any comparative studies on the content of a foodstuff in vitamin B or vitamin G as at present designated raises the question of the balance and

distribution of the factors in the test diet used for the measurements. The results from the first series of experiments on the growth response of rats fed a 2 cc. dose of egg along with different basal diets and vitamin supplements while they showed a satisfactory basis for the estimation of vitamin B and vitamin G, disclosed little evidence of the presence of other factors of the vitamin B complex. Subsequent work may indicate the significance of certain of the differences among the seven dietary treatments. The rice polish extract presumably contains, in addition to the B<sub>1</sub> factor, a B<sub>2</sub> factor necessary for pigeons (21) but not needed by rats or chickens (4). It is possible that yeast autoclaved in an alkaline medium may not retain the B<sub>4</sub> factor (22) which is required by rats. Apparently, egg white left the same deficiency as the alkaline autoclaved yeast. While the growth made by the rats on the combination diet of rice polish extract and alkaline autoclaved yeast was enhanced by the feeding of the 2 cc. dose of egg, it is not certain that the difference between the control and the egg supplemented groups was due to the B<sub>4</sub> and other unrecognized factors or to added supplies of the B<sub>1</sub> and B<sub>2</sub> factors.

While it had been shown previously, as noted, that whole eggs contained both vitamin B and vitamin G, and that egg white was lacking in vitamin B, the experiments in Series II on normal eggs showed that the yolks were relatively richer in vitamin B but that the whole eggs were somewhat richer in vitamin G.

When measured for the relative content of the vitamin B complex in Series 3, the eggs produced on different diets from Groups A and B showed an order of potency comparable to that found in subsequent measurements of vitamin B. The responses of the rats in these tests on the vitamin B complex when rice polish or autoclaved yeast were added to the ration during the third period of the experiment are indicative of the usual observation that whole eggs are richest in vitamin G.

Diet may equalize or even reverse this relationship in the vitamin B and vitamin G content of eggs since a pronounced variation may occur in the former and only a small variation in the latter. Such supplements as rice bran and rice polish rich in vitamin B, when added to hen diets promoted the production of eggs relatively rich in this vitamin but generally with little increase in vitamin G. On the other hand, eggs produced on the deficient diet alone showed approximately half the vitamin B content of normal eggs.

While pronounced differences existed in the potency of the hen diets in both vitamin B and vitamin G, the vitamin G content of the eggs was

apparently not affected to the same extent as the vitamin B content. The observations on the hens and on the hatchability of the eggs are of particular interest in this connection. The hens on the deficient diet of hominy, meat meal, minerals, and cod-liver oil showed a high incidence of polyneuritis during the first four months on experiment.

The birds also laid relatively few eggs, presumably as a result of the dietary. In the late winter and early spring during the usual seasonal period of maximum production, the hens were greatly improved in health. Egg production rose sharply in March of each year but the hatchability remained at a low level since the average for three years was approximately 15 per cent.

Perhaps the most important point in the results from the use of supplements in the deficient ration was the richness of the rice bran and rice polish in vitamin B as compared to yeast. These products were more potent than either of the yeast preparations used in the hen diets. Yet yeast 1 afforded a noticeable benefit to the hens although it did not affect the vitamin B content of the eggs. Yeast 2 was intermediate in effectiveness to yeast 1 and the rice by-products. Both rice bran and rice polish afforded excellent protection to the health of the hens and increased the vitamin B content of the eggs to a level equal to that on normal diets.

While the 15 per cent of rice bran appeared equal, if not superior, to an equal amount of yeast 2 as a vitamin G supplement to the group B basal diet in the rat tests, yeast 2 nevertheless was the more effective of the two as judged by the potency of the eggs.

Apparently the diets of Group A were equally good for the production of eggs of a reasonably high percentage hatchability (3). The vitamin G content of the eggs produced on these diets was not unlike that produced on the Group B diets. Likewise certain of the Group B diets supplemented with feeds especially rich in vitamin B produced eggs equal to those of the Group A diets in vitamin B.

The experiments do not permit of definite conclusions regarding the relation of vitamin B and vitamin G to hatchability. The incidence of polyneuritis among the baby chicks from eggs produced on the Group B basal diet indicated the necessity for this factor. The failure to secure any improvement in hatchability when the yeast or the rice by-products were added to the deficient diet showed that other factors were involved.

While the diet of the hen did not produce especially wide differences in either the vitamin B or the vitamin G content of the eggs, the variations due to the first named factor were nevertheless noteworthy. The lack of

difference in the vitamin G content of the eggs may have been due in part to the relatively low vitamin G content of most, if not all, of the diets.

The richness of eggs in vitamin A was manifested in the results of the 1930-31 experiments. Undoubtedly, the cod-liver oil added to the diets enhanced the vitamin A content of the eggs produced on the normal diets. The deficient diet of Group B yielded eggs richer in vitamin A than generally produced under average conditions although they appeared nearly devoid of yellow pigment.

#### SUMMARY

Two groups of poultry diets, one containing a basal mixture moderately rich and the other a basal mixture deficient in vitamin B and vitamin G which had been found to affect egg production and hatchability, were studied for the effects of the diet of the hen on the vitamin B and vitamin G content of the eggs.

Egg yolks produced on diets adequate in the vitamin B complex contained relatively more vitamin B than vitamin G, while the whole egg was relatively less potent in vitamin B than in vitamin G.

Variations in the vitamin B content of the eggs were produced by the diets fed the hens. Eggs produced on a basal diet of hominy, meat meal, minerals, and cod-liver oil contained from one-third to one-half as much vitamin B as those produced on the same basal diet with rice bran or rice polish incorporated into the mixture or on normal diets containing ground whole cereal grains and mill feeds, with or without protein supplements.

The variations in the vitamin G content were less pronounced. A 2 cc. dose of egg produced weekly gains in rats of 3.8 to 7.9 grams. Although none of the diets was unusually rich in vitamin G, the lack of difference in the eggs was noteworthy in view of the wide difference between the Group A and B diets. In certain cases the vitamin B content was equal to or in excess of the vitamin G content.

The vitamin A content of the eggs was maintained at a high level by the addition of cod-liver oil to both groups of diets, although the basal diet of Group B produced eggs which were low in vitamin B and strikingly deficient in the yellow pigments usually associated with vitamin A.

In the diets studied, the variation or lack of variation in those vitamins determined apparently bore little relation to the hatchability of the eggs.

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# COMPARATIVE EFFECT OF TOMATO AND ORANGE JUICES ON URINARY ACIDITY

By

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IN A previous paper (5) the effect of fresh Malaga grapes, several kinds of grape juice, grape concentrate, and Thompson seedless and Muscat seeded raisins on the urinary acidity was reported. In the experiments with grapes and grape products a 1000 cubic centimeter portion of grape juice or its approximate equivalent of 300 grams of concentrate or raisins was employed. In a subsequent paper (6) the effect of figs and a smaller amount of raisins is given. All of these materials exerted a marked effect on the urinary acidity. Because of their present general use in the diet, it also seemed desirable to ascertain the effect of daily ingestion of similar amounts of tomato and orange juices.

Blatherwick (2) reported that tomatoes produced significant changes in the urine, the pH being increased (decrease of  $H^+$ ) and the ammonia excretion being decreased. His data show that the maximum pH change would be about 0.3 pH unit. In his experiments 200 and 300 grams of tomatoes daily were added to the basal ration. One subject was used to test the effectiveness of each quantity of tomatoes. Apparently fresh whole tomatoes were used in his experiments.

Blatherwick and Long (3) have shown that increasingly large amounts of orange juice produce an increase in the pH of the urine with a decrease in the ammonia content. They used 600 cubic centimeters of juice the first day, increasing the total daily consumption by 600 cubic centimeters each day so that on the fourth or last day 2400 cubic centimeters of juice were taken by each of the two subjects. The pH changes effected by the orange juice were about 0.6 pH unit.

The experiments with the tomatoes (2) did not cause very marked changes in the pH of the urine, nor did they answer the question of the effect of larger quantities of tomato juice of the type such as is now commercially available for table use. Also the orange juice experiments (3) did not show the effect produced by the daily ingestion of some given constant quantity of juice.

The present experiments were of twelve days' duration. Each experiment was divided into two consecutive periods. For the first five days each person received only the basal ration. During the next seven days each received, in addition, 1000 cubic centimeters of either tomato juice or orange juice. Three normal young men subjects were employed in each experiment.

The basal diet was the same as that described in a previous report (5). The 1000 cubic centimeter portions of the juices were used in order that the resulting data would be comparable to those obtained in the previous studies in which 1000 cubic centimeter portions of grape juices were used. A standard commercial tomato juice and the juice from fresh Valencia oranges were employed.

All analytical determinations were made in duplicate or triplicate, the average of closely agreeing duplicates only being reported. The methods were the same as those described in a previous study (5). In addition to the urine analyses, determinations of the ash content, the alkalinity of the soluble ash, the alkalinity of the insoluble ash, and the organic acid content were made upon composite samples of the tomato and orange juices.

### RESULTS AND DISCUSSION

As is shown in Tables I and II, the effect of ingestion of the tomato and orange juices on the pH of the urine is very marked. On the basal diet the pH generally was at equilibrium at 5.80 to 5.70. However, the pH in two instances was lower, being 5.55 for subject R (Table II) and 5.40 for subject N (Table I). The average range compares well with the corresponding range observed in previous experiments (5, 6).

With both the tomato and orange juices there was a generally uniform increase of pH of the urine following their addition to the diet. This increase was such that the pH during the last three days of the experiment was from 0.95 to 1.3 pH units higher than that of the fourth or fifth day of the experiment. The tomato juice produced an average change of 1.2 pH units, while the orange juice caused a change of about 1.05 pH units.

The present results with tomato juice are rather striking when compared to those obtained by Blatherwick (2) with 200 and 300 gram portions of tomatoes. Whereas his maximum pH change of the urine was approximately 0.3 pH unit, the average change in the present experiments was four times as great. This difference very possibly may be largely the result of the ingestion of three to five times the quantity of tomato material. This relation will be discussed in a later paper of this series. Ap-

TABLE I  
EFFECT OF TOMATO JUICE ON COMPOSITION OF URINE

Day*	Subject K					Subject N					Subject O				
	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N
1	1320	6.05	678	43	154	760	5.60	512	175	91	1100	6.30	800	155	140
2	1460	6.05	612	87	139	690	5.45	590	218	99	1190	6.25	681	152	140
3	1100	5.90	540	133	121	580	5.40	509	188	104	890	5.70	587	157	151
4	1110	5.70	525	102	166	720	5.45	540	196	122	980	5.80	510	143	135
5	1160	5.80	544	127	167	840	5.40	590	223	140	740	5.80	523	158	146
6	2060	6.20	705	58	153	1430	6.10	626	186	143	940	5.95	538	55	113
7	1750	6.60	699	56	135	1420	6.20	780	131	128	1590	6.40	652	38	127
8	1820	6.50	625	55	127	1250	6.25	620	63	100	1730	6.50	769	27	123
9	1530	6.70	750	50	106	1180	6.30	635	78	118	1470	6.55	721	27	120
10	1630	6.60	740	55	101	1150	6.50	632	42	92	2300	6.85	896	26	111
11	1900	6.75	788	58	82	1300	6.70	635	43	75	1980	6.80	823	31	108
12	1970	6.70	756	49	66	1690	6.70	788	30	58	1850	7.10†	711	29	95

\* Days, 1-5 inclusive, basal diet.

Days, 6-12 inclusive, basal + 1000 cc. tomato juice.

† Denotes basic urine.



TABLE II  
EFFECT OF ORANGE JUICE ON COMPOSITION OF URINE

Day*	Subject P					Subject Q					Subject R					
	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	Volume	pH	0.1-N Organic acids	0.1-N Titratable acidity	Ammonia N	
1	620	6.00	756	108	191	1060	6.30	571	142	131	670	5.75	537	239	206	mg.
2	770	6.10	730	165	191	980	6.35	586	135	128	550	5.55	504	242	243	cc.
3	690	5.60	702	182	195	820	5.85	535	128	143	590	5.50	520	235	177	cc.
4	900	5.75	635	105	179	1380	5.85	502	130	152	750	5.60	519	232	210	mg.
5	850	5.70	683	99	180	1160	5.85	625	117	136	880	5.55	670	218	185	cc.
6	1260	5.90	739	106	141	1110	5.80	881	109	116	690	5.70	581	114	131	cc.
7	2080	6.35	782	74	108	2050	6.50	817	81	140	1460	6.40	784	83	111	mg.
8	1220	6.50	760	75	105	1850	6.60	847	75	132	1220	6.30	790	92	125	cc.
9	1090	6.50	856	72	113	1410	6.60	809	65	125	1210	6.30	815	97	130	cc.
10	1330	6.65	929	79	105	1660	6.75	823	60	116	790	6.35	711	87	125	mg.
11	1950	6.65	871	57	85	1420	6.75	836	60	106	760	6.50	737	88	110	cc.
12	1730	6.90	761	41	62	1800	6.80	842	61	102	960	6.50	727	82	105	mg.

\* Days, 1-5 inclusive, basal diet.

Days, 6-12 inclusive, basal diet+1000 cc. orange juice.

parently commercially packed tomato juice such as used in these experiments is as effective in increasing the urinary pH as are comparable quantities of grapes, grape products, figs, and orange juice.

A comparison of the present results with orange juice and those of Blatherwick and Long (3) is also of particular interest. With a fourfold increase of orange juice daily ingested during four consecutive days, their data show an average final increase of urinary pH of only 0.5 to 0.6 pH unit, a part of this increase occurring each day. However, the present studies indicate that with a constant daily ration of orange juice the maximum increase was not obtained until the fifth day, the increase then being about 1.05 pH units. Since the 1000 cubic centimeter portions of juice used in the present studies were less than even the 1200 cubic centimeter portions of the second day of Blatherwick and Long's studies, the question may be raised as to what would be the maximum effect produced by the 2400 cubic centimeter portions used by Blatherwick and Long if their experiments had been continued for two or three days longer.

Blatherwick and Long (3) have concluded that the drinking of large amounts of orange juice resulted in the production of alkaline urines. This is obviously not generally or always true. In the present studies an average total increase of urinary pH of 1.05 above that of the basal diet was observed, yet alkaline urines were not obtained. On the contrary, the above workers reported a total pH change of the urine of 0.5 or 0.6 unit above that of the basal diet, or approximately half of that obtained in the present studies. Yet they secured alkaline urines. It therefore seems apparent that the production of alkaline urines in such experiments is not a specific attribute of orange juice. Rather, the production of such alkaline urines is dependent upon the fact that the urinary pH on the basal diet alone was only 0.1 to 0.2 or 0.3 pH unit below neutrality. In such a case any increase, even as small as 0.3 or 0.4 pH unit would, of course, produce the alkaline urines. Consequently, the value of orange juice in lowering urinary acidity may be considerably greater than was indicated by the 0.5 to 0.6 pH unit lowering secured by Blatherwick and Long. That is, subjects with very acid urines may also secure very marked decreases of such acidity (or increase of urinary pH).

The slightly greater increase of urinary pH produced by the tomato juice in comparison to that produced by orange juice is noteworthy when the alkalinities of the ash are considered. While the tomato juice produced the greater change of pH, the ash of the orange juice nevertheless possessed the greater total alkalinity. The total alkalinity of the ash of the tomato

juice was equivalent to 57.2 cubic centimeters of one-tenth normal hydrochloric acid per 100 grams of juice, while that of the orange juice was equivalent to 78.2 cubic centimeters of one-tenth normal hydrochloric acid per 100 grams of juice. However, the alkalinity of the water soluble ash of the tomato juice was equivalent to 47.2 cubic centimeters of one-tenth normal acid, while the corresponding alkalinity of the orange juice was equivalent to 50.75 cubic centimeters of one-tenth normal acid. That is, about 82.5 per cent of the total alkalinity of the tomato juice ash is that of the water soluble ash, while the soluble alkalinity of the orange juice ash is only about 65 per cent of the total alkalinity of the ash. The ratios of soluble ash alkalinity to insoluble ash alkalinity are 4.7 to 1 and 1.85 to 1, respectively, for tomato and orange juices. It is, therefore, possible that not only the magnitude of the total alkalinity but also the ratio of the alkalinities of the water soluble ash and water insoluble ash is important in determining the effect on the reaction of the urine. If such a condition does prevail, it appears that the alkalinity of the soluble ash is more effective in increasing the pH of the urine.

From the data in Tables I and II showing the values for total acids and ammonia excreted daily, it is evident that there was a considerable decrease in their concentrations after both the tomato and orange juices were added to the basal diet.

The evaluation of the quantitative relationship between the carbon dioxide binding power of the blood plasma and the excretion of acid in excess of fixed bases, as measured by determining the ammonia and titratable acid, has been developed by Fitz and Van Slyke (4). In a previous study with grapes and grape products (5) this method of estimating the lowering of the alkaline reserve was employed. It was shown that the basal diet alone lowered the alkaline reserve and the grapes and grape products assisted in increasing the alkaline reserve to a point above its previous level. From a comparison of the data in Tables I and II with those of the previous study, it is evident that both tomato and orange juices increased the alkaline reserve of an individual even above the normal as estimated from the data of the first day of an experiment. This was indicated approximately by the considerably smaller values for the ammonia and titratable acidity titrations. The raising of the alkaline reserve was of the same order of magnitude, or slightly greater, as was that produced by equivalent quantities of grapes and grape products.

The relation between the percentage oxidation of the organic acids and the changes of the pH are of interest. The organic acid content of the to-

mato and orange juices was determined by the method of Van Slyke and Palmer (7). The total amounts daily ingested (as cubic centimeters of tenth-normal acid) are given in Table III. The differences of the average daily organic acid titration of the urine with and without the juices added to the basal diet are also given. From these values the percentage of the organic acids oxidized in the body may be computed. An average of 90.7 per cent of the organic acids of the tomato juice was oxidized while an average of 93.8 per cent of the organic acids of the orange juice was oxi-

TABLE III  
ORGANIC ACIDS INGESTED AND OXIDIZED  
(As cc. N/10 HCl)

	Tomato Juice			Orange Juice		
	K	N	O	P	Q	R
Average daily organic acid titration for basal diet period.....	580	548	600	701	564	550
Average daily organic acid titration for basal diet+tomato and orange juice.....	723	673	733	814	836	735
Difference.....	143	125	133	113	272	185
Organic acids ingested daily from tomato or orange juice.....	1450	1450	1450	3087	3087	3087
Per cent oxidation of organic acids....	90.1	91.1	90.8	96.3	91.2	94.0

dized. However, the tomato juice produced a somewhat greater change of urinary pH. If the organic acids do influence the pH, as is possible, this difference of effect may be in part the result of the higher organic acid content of the oranges. The data in Table III indicate that the organic acid content of the orange juice used in these tests was slightly more than twice as great as that of tomato juice. Hence the final average amount of organic acids left unoxidized from the orange juice may be somewhat larger than that from the tomato juice. Using the data in Table III it appears that, expressed as cubic centimeters of tenth-normal acid, about 135 parts of the organic acids of tomato juice and 190 parts of the organic acids of orange juice are not oxidized. It is therefore probable that the unoxidized organic acid residue is a limiting factor in relation to the magnitude of the pH change of the urine in such experiments.

The present value of 93.8 per cent oxidation of organic acids of orange juice compares well with that of 94 per cent reported by Blatherwick and Long (3) for orange juice.

## SUMMARY

Experiments with men subjects on a basal diet and on the same basal supplemented by tomato and orange juices are reported. The following results were observed when the juices were added to the basal ration.

1. An average increase of the urinary pH of 1.20 pH units was produced by 1000 cubic centimeters of tomato juice taken daily. An equal quantity of orange juice produced a similar average increase of 1.05 pH units.

2. Corresponding decreases in both the ammonia excreted and in the total acidity were noted. The average changes produced by the two juices were approximately the same.

3. There was an increase of the alkaline reserve, calculated according to the method of Fitz and Van Slyke, above the normal for each subject. This increase was quite marked for both juices.

4. There appeared to be a correlation between the alkalinity of the ash and the reaction of the urine. A more basic reaction was associated with the higher ratio of soluble alkalinity to insoluble alkalinity of the ash. The tomato juice exhibited the higher ratio and produced a somewhat larger change in reaction.

5. An increase occurred in the organic acids excreted when either tomato or orange juice was added to the basal ration.

6. The average oxidation of the organic acids of tomato juice was 90.7 per cent while that of orange juice was 93.8 per cent.

The writers wish to express their appreciation of the interest and advice of Dr. W. V. Cruess, upon whose suggestion this study was initiated. The full coöperation of the men taking a part in the diets is gratefully acknowledged.

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## THE EVALUATION OF THE PHOSPHORUS DEFICIENCY OF THE RICKETS- PRODUCING DIET

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WE were eager to evaluate the extent of the deficiency in the rickets-producing diet of Steenbock and Black no. 2965 (1). This contains an excess of calcium and a deficiency of phosphorus. That phosphorus intake may be a limiting factor in growth has been shown by Osborne and Mendel (2). Upon a diet of 18 per cent of either edestin or casein the growth of rats was inhibited, but resumed when phosphate was added. They cite this as an example of Liebig's law of the minimum. Their experiments antedated the discovery of vitamin D; but the diets contained 18 per cent of butter and therefore must have included considerable vitamin. The contrary has been reported by Bethke, Steenbock and Nelson (3) who state<sup>1</sup> that "with casein as the protein, fed at a level of 18 per cent of the ration, the phosphorus cannot be sufficiently reduced to cause nutritive failure. Even with lower levels of cod liver oil intake, increase of phosphate does not lead to increased growth."

It is well known that the calcium and phosphorus metabolism are interdependent. An increase in the calcium may cause a relative insufficiency of the phosphorus. Examination of the calcium and phosphorus content of the diets given in Table I shows that the phosphorus of the diet of Bethke, Steenbock and Nelson lies between that of the edestin and casein diets of Osborne and Mendel and that the calcium is essentially the same. In the rickets-producing diet No. 2965, the phosphorus which is considered below the optimal for rats is higher than in the low phosphorus diets, but the excessive calcium makes it relatively low.

Rats fed the rachitogenic diet No. 2965 of Steenbock and Black, which contains a moderate amount of vitamin A and is deficient in D, gain in weight for only 3 to 4 weeks. After that they decline in weight and die in

<sup>1</sup> Reference 3, page 283.

TABLE I

Author	Diet	Calcium	Phosphorus	Ca:P
		mg. per cent	mg. per cent	
Osborne and Mendel (1)	Edestin 1	500	35	14
Osborne and Mendel (2)	Casein 2	500	169	3
Osborne and Mendel (2)	Control 1	546	372	1.4
Osborne and Mendel (2)	Control 2	546	511	1
Bethke, Steenbock, and Nelson (3)	Low phosphorus	447	55	8
Steenbock and Black (1)	No. 2965	1250	250	5

9 to 12 weeks. Hence such animals are unsuitable for investigation of the effect of phosphorus deficiency. The addition of cod liver oil to the diet prevents the development of rickets or cures it. Bethke, Steenbock, and Nelson (3) have shown the interrelationship of calcium-phosphorus imbalance and vitamin intake on growth. With increase of vitamin, the growth is improved for a given calcium deficient diet. Thus we hoped without changing the mineral content of the diet to maintain the animals in good condition and growth for a long time.

In addition to the growth, other criteria of metabolism are available: x-ray pictures, the analysis of the blood serum, bones, and tissues, and the measurement of the intake and output of calcium and phosphorus.

When rats are fed a high calcium low phosphorus diet such as No. 2965 of Steenbock and Black, whether or not vitamin D is also fed, a relatively high calcium low phosphorus retention results, though both retentions are below normal. These findings are based upon short-time studies (1 to 5 weeks) (4, 5). The question at once arises as to what the ultimate outcome of such a regimen would be. Does the type of retention change so that the calcium-phosphorus balances no longer reflect the proportions present in the food, or does the body composition, especially that of the bones, become altered?

#### EXPERIMENTS

Animals were bred from the Babies and Childrens Hospital stock colony of albino rats of Wistar Institute stock. The mothers were fed Sherman's diet B (6) consisting of whole wheat  $\frac{2}{3}$  and whole milk powder  $\frac{1}{3}$  plus 1.33 per cent NaCl. In addition they were given meat and liver, about 5 gm. each, and lettuce once a week. While pregnant they were also given 400 mg. of compressed yeast tablet daily.

The young were weaned at 21 days and continued on Sherman's diet without any additions for seven days. At the age of 28 days they were placed upon the experimental diet. This consisted of Steenbock and Black's diet No. 2965—76 per cent ground yellow corn, 20 per cent gluten flour, 3 per cent  $\text{CaCO}_3$  and 1 per cent  $\text{NaCl}$ . To each 100 gm. of diet 2 gm. of cod liver oil were added. The diet was made fresh every week. The experiment was run in 1930 and repeated in 1931. Metabolism studies were made for 7-day periods, during the 5th, 6th, and 7th weeks the first year and 9th, 14th, and 19th weeks the second year. It was feared that the animals in the metabolism cages would not be strictly comparable in growth to those with easier access to food and more ample room for exercise, provided by our stock cages. Therefore in the first series the metabolism was studied for only 3 weeks. For the remaining 17 weeks they were kept in the screened stock cages without bedding. In the second series the control and experimental animals were confined to the metabolism cages throughout the experimental period and were controlled by litter mates in stock cages on both the experimental and standard diets.

The metabolism cages which caused separation of urine and feces were cleaned daily with distilled water and a rubber policeman. The food was analyzed for calcium and phosphorus and, from the weight of that consumed, the intake of these elements was calculated. The food spilled was separated from the excreta, and deducted from intakes. In these experiments it was so small as to be practically negligible. Only a small portion was placed in the feeding cup at one time; the small amount remaining each day was thoroughly mixed with the new portion, to insure consumption of all constituents. The urine and feces were analyzed for calcium and phosphorus. At the age of 170 days in the first experiment and 133 days in the second experiment the animals were x-rayed, etherized, and killed by bleeding. Litter mates, fed the diet B of Sherman, served as controls.

*Analytical methods.* The blood serum was analyzed for calcium by the method of Clark and Collip (7), and for serum inorganic phosphorus by the method of Kuttner and Cohen (8). The femurs were dissected, dried, extracted with alcohol and ether, and the ash determined (3). The ash of the femurs was also analyzed for calcium and phosphorus. The remainder of the rat was incinerated and ashed. The ash was dissolved in  $\text{HCl}$ , and calcium and phosphorus determinations were made upon aliquots. The calcium was precipitated as the oxalate after adjustment to pH 5.0 to 5.6 and titrated with permanganate. The phosphorus was precipitated as the molybdate, treated with formaldehyde, and titrated with  $\text{HNO}_3$  and



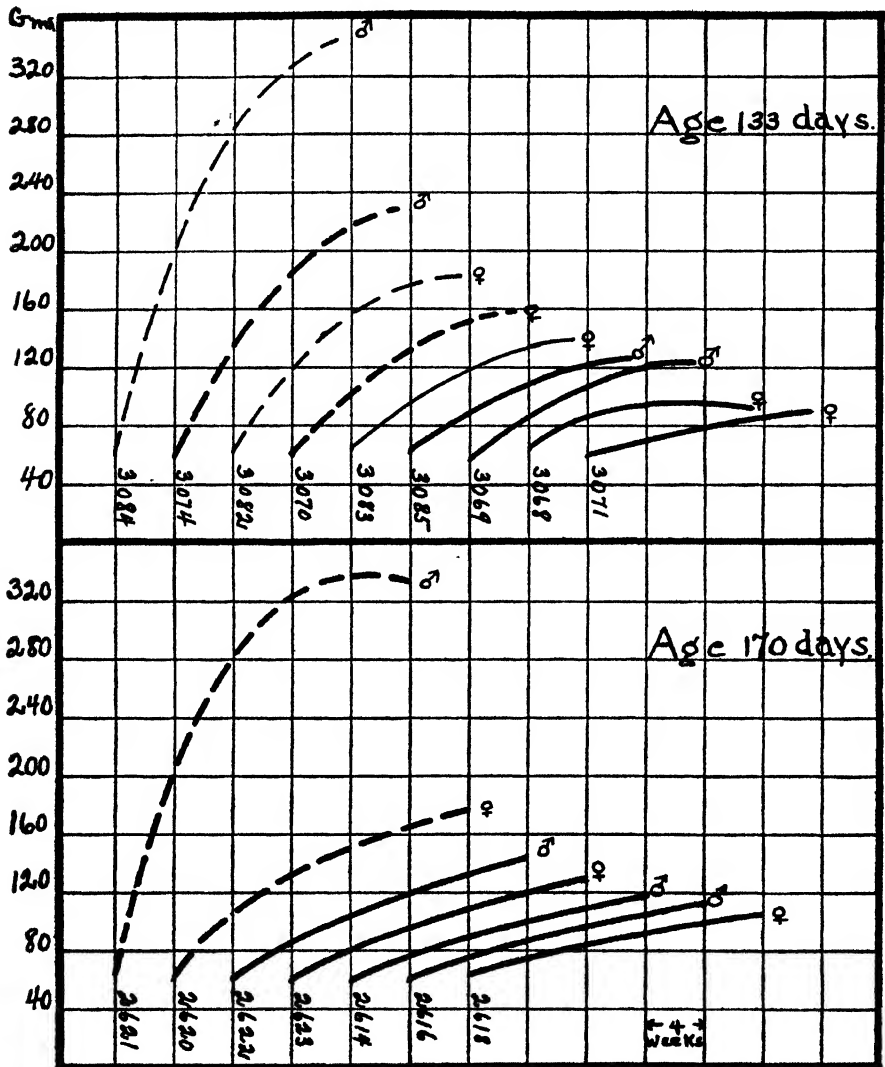


FIG. 1.—The solid lines represent growth curves of animals fed Steenbock and Black's diet No. 2965; the broken lines, the growth curves of those fed Sherman's diet B. In the upper section, the light lines refer to rats in stock cages and the heavy lines to those in metabolism cages throughout the experiment. In the lower section all curves represent the animals which were in metabolism cages for only the 5th, 6th and 7th weeks. All weight curves begin at 28 days of age.

NaOH. The accuracy of the methods of tissue and food analysis was of the order of 1 per cent.

*Progress.* The experiment proceeded with no known errors. All the animals appeared normal, except one that died of acute retention of urine and was discarded. The fur and eyes were in good condition. The activity of the experimental animals was similar to that of the controls. They appeared normal except that they were slenderer and smaller.

## RESULTS

*The x-ray pictures* showed that no rickets developed and, by this criterion, the bones were normal but smaller and thinner than those of the control animals.

*The increase in weight* of the experimental animals is charted in Fig. 1. This shows clearly that the experimental animals were definitely stunted compared to the normal controls. The weights of both males and females were below those of the control females. Whereas for both series the control males gained 12 to 13 gm. a week, the control females increased 6 to 7 gm. weekly and the experimental males and females averaged 4 and 2.5 gm., respectively, i.e., only 30 to 40 per cent of normal growth.

It is clear from Fig. 1 that at 133 days the animals in the metabolism cages did not gain as much as those in the stock cages. However, the same relations between diet and growth are evident whether under similar or dissimilar conditions. The changes which this alteration in weight brought about will be manifest when the analysis of the bodies is discussed.

We have previously shown that the experimental diet permits good growth when phosphate is added (9). Therefore the conclusion must be drawn that the phosphorus deficiency is the limiting factor in the growth.

*The blood serum* content of calcium of both normals and controls throughout the period investigated remained at the level of 10 mg. per cent. The inorganic phosphorus of the serum, as in humans, diminishes at maturity. In the first few months of life on the control diet values of 8 to 10 mg. per cent were found; at 130 to 135 days 6 to 7 mg. per cent and at 170 days 5 mg. per cent; the experimental animals at the same time showed 4.7 and 4.0 mg. per cent, respectively. Thus on the low phosphorus diet, even though cod liver oil was consumed, the phosphorus value was slightly lower than that found in the controls, but above the level found in rickets.

*The bone ash.* The ash percentage of the extracted femurs given in Table II shows 63.5 per cent at 133 days for the normals (average of males and females) and 56.2 per cent for the experimental animals; at 170 days 61.0

TABLE II  
BONE ANALYSIS PER SINGLE FEMUR

Rat No.	Sex	Wet wt.	Dry wt.	Fat-free wt.	Ash of fat-free bone						
		mg.	mg.	mg.	mg.	per cent	Calcium		Phosphorus		Ca: P
							mg.	per cent	mg.	per cent	
Age 133 days. Sherman's diet B.											
3074*	♂	633	435	390	247	63.4	95	38.5	44	17.8	2.16
3084†	♂	804	546	499	319	63.9	118	37.2	55	17.2	2.15
3070*	♀	440	330	305	189	62.2	74	39.0	34	18.0	2.17
3082†	♀	522	377	342	217	63.6	82	37.8	39	18.0	2.11
Age 133 days. Steenbock and Black's diet No. 2965, plus cod liver oil.											
3069*	♂	387	258	222	116	52.6	45	38.6	20	17.3	2.25
3085*	♂	403	237	206	116	56.2	42	36.2	20	17.3	2.10
3068*	♀	342	225	196	107	54.9	43	40.0	19	17.8	2.26
3071*	♀	278	200	172	98	57.2	37	37.8	18	18.4	2.06
3083†	♀	416	288	255	154	60.5	59	38.2	27	17.5	2.18
Age 170 days. Sherman's diet B.											
2621†	♂	1040	676	652	406	62.2	157	38.7	74	18.2	2.13
2620†	♀	585	384	364	218	59.8	85	38.9	40	18.3	2.12
Age 170 days. Steenbock and Black's diet No. 2965, plus cod liver oil.											
2614†	♂	419	255	233	131	56.3	49	37.5	24	18.3	2.04
2616†	♂	420	256	224	118	52.8	46	39.0	21	17.8	2.19
2622†	♂	500	316	288	161	55.8	65	40.3	29	18.0	2.19
2618†	♀	420	271	245	143	58.4	54	37.8	25	17.5	2.16
2623†	♀	480	313	298	162	54.4	64	39.4	29	17.9	2.20

\* In metabolism cage for duration of experiment.

† In stock cage.

‡ In metabolism cage for 5th, 6th, and 7th weeks of life.

per cent for the controls and 55.5 per cent for the experimental animals. The values for the normals are in agreement with those found by other investigators but those of the experimental animals are too low to be classed as normal and too high to fall in the zone of values found in rachitic animals.

At 133 days the animals in the metabolism cages were smaller than

those in the stock cages and had lighter bones. The actual weights of the bones of the experimental animals, whether fresh, dry, or extracted, or of the ash, calcium, and phosphorus, were about 55 to 60 per cent of the normal. Closer analysis shows that, compared to the controls, the bones of the experimental rats were actually increased in fat, relatively richer in water and organic material and poorer in ash.

At 170 days the same general relations held except that the stunting effect of the rachitogenic diet was more marked and here the values of the bones reached only 30 to 40 per cent of that of the controls. Thus the growth of the femur closely resembles the body increase in weight. The bones of both the experimental and control groups comprise the same fraction of total weight.

A considerable amount of data has accumulated as to the composition of the ash of the bones in respect to the proportion of calcium to phosphorus. The whole question of the physical chemistry of bone formation and the possible mechanism involved has depended upon the composition of bone. We have heretofore merely determined the total ash, on the assumption that the composition with respect to calcium and phosphorus was constant within the limits of accuracy required. In this experiment, because of the long duration on low phosphorus diet, this hypothesis was subjected to a very severe test. It seemed desirable therefore to determine calcium and phosphorus separately. Analysis of the ash of the bones of the control animals, extracted with alcohol and ether, at 133 and 170 days of age gave approximate values of 38 per cent calcium and 18 per cent phosphorus with a Ca:P ratio of 2.14. The experimental animals, male and female, gave values which were the same, within the errors of the methods. Thus one must conclude that the mineral salts of bone have constant composition in respect to Ca:P even under the stringent conditions of this experiment. This is the second example of the law of the minimum. In this case only so much bone is formed as is permitted by the phosphorus content of the diet.

*Metabolism studies.* The intake of calcium and phosphorus of animals fed Sherman's diet B differs from that which we have previously reported normal with other diets (9). This diet has a Ca:P of 0.6 to 0.7 and is therefore a high phosphorus diet. We have previously discussed the significance of this factor in the diet and come to the conclusion that in a normal diet the Ca:P should be between 1 and 2, probably between 1.5 and 2 and that 2 was the best value available.

The data for Sherman's diet B for the 6th, 9th, 14th, and 18th weeks of

**TABLE**  
**METABOLISM OF CAL**  
**Figures in terms of**

Rat No.	Sex	Age	Calcium					
			Intake	Urine	Feces	Total output	Retention	
		weeks	mg.	mg.	mg.	mg.	mg.	per cent

Sherman's

2632	♂	5-7	272	7	11	18	254	93
2634	♂	5-7	230	6	12	18	212	92
2637	♂	5-7	256	5	13	18	238	93
Av.	♂	5-7	253	6	12	18	235	93
2631	♀	5-7	233	7	8	15	218	94
2633	♀	5-7	212	6	7	13	199	94
2638	♀	5-7	226	8	15	23	203	90
Av.	♀	5-7	224	7	10	17	207	93
3074	♂	8-9	305	7	58	65	240	79
3070	♀	8-9	243	8	38	46	197	81
3074	♂	13-14	191	6	117	123	68	36
3070	♀	13-14	180	5	76	81	99	55
3074	♂	18-19	173	6	149	155	18	10
3070	♀	18-19	177	5	117	123	54	30

Steenbock and Black's diet

Av.*	♂	4-5	586	90	390	480	106	18
Av.†	♀	4-5	570	72	412	484	86	15
Av.*	♂	5-6	662	102	272	374	288	43
Av.†	♀	5-6	759	51	267	318	441	58
Av.*	♂	6-7	597	84	292	376	221	37
Av.†	♀	6-7	684	106	339	445	239	35
3069	♂	8-9	492	159	193	352	140	28
3085	♂	8-9	468	112	223	335	133	28
3068	♀	8-9	684	197	301	498	186	27
3071	♀	8-9	480	107	172	279	201	42
3069	♂	13-14	397	120	173	293	104	26
3085	♂	13-14	508	143	255	398	110	22
3068	♀	13-14	397	102	167	269	128	32
3071	♀	13-14	422	104	205	309	113	27
3069	♂	18-19	437	140	299	439	-2	—
3085	♂	18-19	578	177	322	499	+78	13
3086	♀	18-19	519	205	261	466	+53	10
3071	♀	18-19	437	175	329	504	-67	—

\* Rats no. 2614, 2616, 2622.

† Rats no. 2618, 2623.

### III

#### CALCIUM AND PHOSPHORUS

one rat per week

Phosphorus						Retention Ca:P
Intake	Urine	Feces	Total output	Retention		
mg.	mg.	mg.	mg.	mg.	per cent	

#### Diet B.

391	124	59	183	208	53	1.22
332	121	50	171	161	48	1.32
370	72	59	131	239	65	1.00
364	106	56	162	202	55	1.16
335	118	47	165	170	51	1.28
306	111	43	154	152	50	1.31
325	124	46	170	155	48	1.31
322	118	45	160	162	49	1.28
475	114	92	206	269	57	0.89
378	89	81	170	208	55	0.95
297	99	110	209	88	89	0.77
280	66	67	133	147	52	0.67
267	91	116	207	60	30	0.30
274	60	93	153	121	44	0.45

#### No. 2965, plus cod liver oil. ‡

123	5	83	88	35	28	3.03
120	5	80	85	35	24	2.46
139	5	79	84	55	40	5.24
160	5	80	85	75	47	5.88
126	5	78	83	43	34	5.14
144	5	83	88	56	39	4.26
121	4	65	69	52	43	2.70
115	4	64	68	47	41	2.83
168	4	81	85	83	49	2.24
118	4	51	55	53	45	3.80
75	9	54	63	12	16	8.70
96	9	65	74	22	23	5.00
75	9	47	56	19	25	6.70
80	9	54	63	17	21	6.60
85	4	88	92	-7	—	—
113	4	88	92	+21	19	3.70
101	4	86	90	+11	11	4.80
85	4	85	89	-4	—	—

‡ Rats were weaned at 21 days of age, and continued on Sherman's diet B until 28 days of age, when the experimental diet was begun.

life, given in Table III, show several interesting features. The food intake was maximal at the 9th week. Throughout these periods the calcium excretion in the urine was nearly constant; 5 to 8 mg. per rat per week, or 2 to 3 per cent of the intake. Differing from our previous experience with other normal diets (with a  $\text{Ca:P} = 2.0$ ), the urinary calcium was in all cases less than the phosphorus.

The fecal calcium was also low throughout but especially in the period of the 6th week. Here the calcium in the feces represents less than 5 per cent of the intake. Again differing from our previous experience with other normal diets relatively higher in calcium, the fecal calcium was less than the fecal phosphorus for the first two periods. For the last two periods the calcium in the feces was greater than the phosphorus. As the fecal calcium increased with age, the fecal phosphorus also rose and smaller percentages of the calcium and phosphorus were found in the urine. The small amount of calcium in the urine and feces might be expected to result in high  $\text{Ca:P}$  retention. But such is not the case. Although during the 6th week 93 per cent of the calcium was retained, this dropped to 80 per cent in the 9th week, 36 to 55 per cent in the 14th week, and 10 to 30 per cent in the 19th week. The retention of the phosphorus remained at about 50 per cent throughout the period of observation. This resulted in diminishing ratios of  $\text{Ca:P}$  retained. Even for the first period the  $\text{Ca:P}$  ratios were only 1.16 and 1.28. We have considered 1.5 to 1.7 as normal at that age. The rest of the values, which are below 1.0, represent *high phosphorus* retentions.

Upon the experimental diet with a  $\text{Ca:P}$  of 4.0 to 5.3 the findings are in contrast to those for the controls and similar to our previous experience with high calcium low phosphorus diets. The urine was nearly phosphorus free and the fecal calcium was far greater than the fecal phosphorus in every case. The percentage of retention and the actual retention of both calcium and phosphorus were greater during the earlier periods of study than at the 14th and 19th weeks. In the last period negative balances of calcium and phosphorus were found in two cases.

The  $\text{Ca:P}$  retentions show consistently high ratios throughout, similar to those observed in the course of development of rickets, and represent, except in the last period where negative balances occurred, an *excess calcium* retention. In general the type of retention reflects the relation of minerals in the diet.

*Analyses of whole animals.* Whereas metabolism studies should indicate what is occurring during the period of study, the ash of the whole body should give information about the summation of all that has gone before.

Sherman and Booher (10) have recently used the ash method for biological assays of various types of diets, especially those of low and varying calcium content. Sherman and Quinn (11) have studied also the phosphorus content of male and female rats fed diet B for a long time. McCann and Barnett (12) have analyzed the bodies and bones of rats fed for four weeks a rickets-producing diet with and without vitamin D.

The values for the body content for calcium and phosphorus, shown in Table IV, were obtained by adding the amount found in the femurs and

TABLE IV  
ANALYSIS OF TOTAL BODY FOR CALCIUM AND PHOSPHORUS

Rat No.	Sex	Diet§	Net body					
			Weight	Calcium		Phosphorus		Ca:P
				gm.	gm.	per cent	gm.	
Age 133 days.								
3074*	♂	Sherman	206	2.54	1.23	1.42	0.69	1.79
3069*	♂	Steenbock	108	1.30	1.20	0.74	0.69	1.76
3085*	♂	Steenbock	111	1.29	1.16	0.76	0.69	1.70
3070*	♀	Sherman	143	2.12	1.48	1.14	0.80	1.86
3068*	♀	Steenbock	83	1.29	1.56	0.73	0.88	1.77
3071*	♀	Steenbock	79	1.18	1.49	0.67	0.85	1.76
3084†	♂	Sherman	315	3.07	0.98	1.74	0.55	1.76
3082†	♀	Sherman	166	2.11	1.27	1.16	0.70	1.82
3083†	♀	Steenbock	128	1.63	1.27	0.92	0.72	1.77
Age 170 days.								
2621†	♂	Sherman	294	3.40	1.16	1.99	0.68	1.71
2614†	♂	Steenbock	104	1.38	1.32	0.76	0.73	1.82
2616†	♂	Steenbock	105	1.34	1.28	0.74	0.71	1.82
2622†	♂	Steenbock	132	1.66	1.26	0.94	0.71	1.77
2620†	♀	Sherman	156	2.28	1.46	1.27	0.81	1.80
2618†	♀	Steenbock	98	1.50	1.53	0.80	0.82	1.87
2623†	♀	Steenbock	115	1.71	1.49	0.94	0.82	1.82

\* In metabolism cage for duration of experiment.

† In stock cage.

‡ In metabolism cage for 5th, 6th, and 7th weeks of life.

§ The diets were Sherman's diet B and Steenbock and Black's diet No. 2965 plus cod liver oil.



that in the remainder of the body. They show the well-known sex differentiation—a greater per cent of minerals occurs in the females. The control rats in the stock cages were heavier not only than those in the metabolism cages but also than Sherman's animals (11) and show a slightly smaller per cent of both calcium and phosphorus. If these analyses were on a fat-free basis, closer agreement would probably result. The principal deduction to be drawn, in agreement with McCann and Barnett, is that *the ratio of total body calcium to phosphorus is essentially the same for males and females, experimental and control, on the high phosphorus and low phosphorus diets.*

This is an extension of the two previous examples of the law of the minimum. Only so much growth is permitted as allows the composition of similar (but not necessarily constant) structure. Minor differences which our methods do not reveal as significant differences, may be present. Another law may be operative. We know that tissues and body fluids may vary in certain minor but important respects (such as to justify the determinations we make daily in the laboratory for the diagnosis of disease). Such observations have been made in rickets recently by Haury (13) and Cole and Koch (14).

However, if one assumes that 99 per cent of the calcium is in the skeleton, the phosphorus associated with it can be computed. If one assumes a constant relation of Ca:P in all the bones, the total calcium in the body, divided by 2.14, equals the phosphorus in the bones. The remaining phosphorus is that distributed throughout the body. We have made such calculations and find no significant differences in the phosphorus of the soft parts of the rats fed the two diets. We have analyzed the tissues of rats on high calcium low phosphorus diets with and without vitamin D and have found no difference in phosphorus by the methods we used.

How then can the results of the metabolism and the analyses be compared? By the former methods the rats on the high calcium low phosphorus diets continue throughout the experimental period to have high calcium low phosphorus retentions. By the latter methods the composition of the body in respect to per cent of calcium and phosphorus and hence in their proportion to each other is normal. The results of the two methods are divergent. The explanation presents an unsolved problem.

The experimental animals are still laying down bone as rapidly as the limiting phosphorus in the diet permits, whereas the normal animals have reached complete development of their skeleton and have no longer a need for extra calcium. As far as bone deposition is concerned, the experimental animals are growing and the normal animals are adult.<sup>1</sup>

<sup>1</sup> Footnote p., 283.

### SUMMARY

On a high calcium low phosphorus diet, in which the moderate content of vitamin A and deficiency in D is supplemented by vitamins A and D, growth is retarded. The metabolism continues for 19 weeks to show a high calcium low phosphorus retention.

The fat-free bones are normal in respect to the relative amounts of calcium and phosphorus which they contain.

The body shows no alteration from the normal in respect to per cent of calcium and phosphorus and their relative proportion.

The phosphorus deficiency of this diet causes delayed growth and is the limiting factor.

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<sup>1</sup> After the manuscript was sent to the editors, the article by Kletzien, Templin, Steenbock, and Thomas, entitled "Vitamin D and the Conservation of Calcium in the Adult. I," appeared in the *Journal of Biological Chemistry*, **97**, p. 265. In their experiments they also used Diet No. 2965, alone and with Vitamin D supplied by irradiation; pregnancy and lactation were used as additional stress instead of growth. They state further, "On this same ration, we have observed a distinct tendency to the production of bones of lower ash content with young growing rats even when they received an abundance of vitamin D." Our work thus confirms their finding, and adds evidence of a different type.

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# THE SPECTROGRAPHIC ANALYSIS OF MILK ASHES

By

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IN RECENT years considerable attention has been focused upon the physiological and nutritional significances of the so-called biologically rare elements. It is only natural, therefore, that milk should be among the materials examined for the presence or absence of inorganic elements which are biologically rare or not among the major inorganic constituents of living matter. Accordingly, Wright and Papish (1) made spectrographic analyses of a number of cow's milks from the United States and Great Britain. As a result they concluded that milk contains the following elements in relatively large quantities: calcium, magnesium, phosphorus, potassium, and sodium; and the following elements in smaller concentrations or "traces": aluminum, boron, copper, iron, lithium, manganese, silicon, strontium, titanium, vanadium, zinc, and very likely rubidium.

More recently Zbinden (2), also using the spectrographic method, analyzed a number of cow's milk and human milk ashes from widely distributed European sources. From his results he concludes that both cow and human milk contain regularly and in approximately constant although small quantities the following elements: aluminum, chromium, copper, iron, manganese, lead, tin, titanium, vanadium, and zinc. To this list he adds silver as a probable constituent of human milk.

The results of these two investigations agree with respect to seven elements, viz.: aluminum, copper, iron, manganese, titanium, vanadium, and zinc, but disagree with respect to eight other elements in that Wright and Papish report boron, lithium, rubidium, silicon, and strontium, which are omitted by Zbinden (possibly because the most sensitive lines of some of these elements were not in the spectral region examined), whereas Zbinden reports chromium, lead, and tin which Wright and Papish omit. In view of this disagreement it seems warranted to publish a third set of data on the biologically rare elements contained in milk.

### MATERIAL

The materials<sup>1</sup> used in this study consisted of 19 samples of cow's milk. Sixteen of these were samples delivered to the laboratory in standard commercial one quart bottles of dairies located in Baltimore, Philadelphia, Pittsburgh, New York City, and the District of Columbia. Of these 16 samples 8 were labeled pasteurized, 3 unpasteurized, and 5 certified. Presumably all were regular commercial stock products. The remaining 3 samples were milks of 3 different cows of a certified milk-producing herd and were obtained by milking directly into previously acid cleaned and distilled water rinsed glass-stoppered pyrex bottles under conditions prevailing in certified milk-producing dairies. A sample of the feed mixture fed to these certified milk-producing cows was also obtained and analyzed.

### METHODS OF ANALYSIS

The methods of analysis were spectrographic and therefore fundamentally the same as those used by Wright and Papish and by Zbinden. Approximately ten gram portions of the materials, viz., the 19 milks and the feed mixture, were ashed at low temperature in both silica and platinum dishes directly over Bunsen burners in a relatively small room used only for that purpose at the time and therefore free from other fumes. During the ashing the room was furthermore kept as free as possible from air currents. Contamination of the samples seemed less likely by this procedure than by ashing in a muffle furnace. Immediately after ashing, the dishes containing the ashes were placed underneath glass cover dishes and stored in this condition until subjected to spectrographic analysis.

The technic employed in the spectrographic analysis of these ashes was essentially that described by Tourtellotte and Rask (3) except that graphite electrodes were used in addition to copper electrodes, and examinations were made of the spectra extending from 2400 to 6800 Ångström Units. The spectrograph used was the Hilger E-1, an instrument of particularly good resolving power throughout the ultraviolet and short visible regions. A total of at least three spectrograms was prepared of each ash. These spectrograms included, in the case of every ash, arc spectrograms with graphite and spark spectrograms with copper electrodes. These spectrograms were searched for "*raies ultimes*" of the elements listed in the following summary, which omits elements such as chlorine, iodine, etc. which are not detectable by ordinary spectrographic methods:

<sup>1</sup> Kindly supplied by Dr. J. H. Shrader, Director, Research Laboratories of the National Dairy Products Corporation.

## SUMMARY

1. The following elements were found in large quantities in all milk samples: calcium, magnesium, phosphorus, potassium, and sodium.

2. The following elements were found in smaller quantities (traces) in all milk samples: barium, boron, copper, iron, lithium, rubidium, strontium, titanium, and zinc. Of these barium is the only one which has not been reported previously.

3. The presence of aluminum and manganese was not definitely demonstrated, although these elements were present in the feed mixture.

4. The presence or absence of silicon and vanadium could not be established definitely owing to the presence of these elements in the graphite electrodes used.

5. The presence of antimony, arsenic, bismuth, caesium, cobalt, cadmium, fluorine (as the  $\text{CaF}_2$  band), germanium, gold, indium, lanthanum, mercury, molybdenum, nickel, osmium, palladium, silver, zirconium, chromium, lead, and tin could not be detected, although Zbinden concludes that the last three of these elements, viz., chromium, lead, and tin, are present in European milks.

The following table shows how these results compare with those of Wright and Papish and those of Zbinden, omitting calcium, magnesium,

MINERAL ELEMENTS IN MILK AS DETECTED BY THE INDICATED INVESTIGATORS

	Wright and Papish	Zbinden	Blumberg and Rask
Aluminum.....	+	+	?
Barium.....	-	*	+
Boron.....	+	*	+
Chromium.....	-	+	-
Copper.....	+	+	+
Iron.....	+	+	+
Lead.....	-	+	-
Lithium.....	+	*	+
Manganese.....	+	+	?
Rubidium.....	+	*	+
Silicon.....	+	-	?
Strontium.....	+	*	+
Tin.....	-	+	-
Titanium.....	+	+	+
Vanadium.....	+	+	?
Zinc.....	+	+	+

+ Present  
- Absent

? Questionable  
\* Not tested for

phosphorus, potassium, and sodium, concerning whose presence in milk there is no question.

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## STUDIES ON THE ROLE OF ZINC IN NUTRITION

By

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THE simplest chemical composition of the complete diet of man and the higher animals is a subject of great theoretical as well as practical interest. The total number of pure chemical substances needed is not, as yet, known with certainty. Studies on nutrition since the earlier years of this century have disclosed the subtle influence of almost unbelievably small amounts of materials in the diet on the health of the animal and even the maintenance of life itself. The discovery and study of vitamins has done much to promote research toward the goal of successfully rearing animals on a diet the chemical composition of which is definitely known.

The field of study of the inorganic constituents of foods has been overshadowed by the interest in the study of vitamins; however, recent studies on the effect of the deprivation of certain elements have led to interesting results. Thus, copper (1) and manganese (2) have been found to be essentials for the complete diet, while fluorine (3), aluminum (4), and nickel (5) have been found to be dispensable.

Zinc, though one of the less usual elements, is almost universally present in plant and animal substances. Investigators who have examined carefully enough for its presence have reported zinc in a great variety of biological materials. "Man ingests in his food practically as much zinc as he does iron." (6) This would tend to indicate that zinc has some nutritional function. It is, therefore, an interesting question as to what would happen to an animal deprived of zinc during its lifetime.

This question has been studied before but without conclusive results. Bertrand and Benson (7) conducted feeding experiments on mice with a zinc-free diet. This diet was composed of starch, casein, cellulose, lactose, cocoa butter, and a salt mixture. It lacked all the vitamins and, naturally, the animals failed. Animals fed on this diet lasted about 15 to 20 days,

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while others fed the same ration with the addition of one hundredth of a gram of zinc per hundred grams of food lived 25 to 50 per cent longer.

McHargue (8) tried to find out whether growth would take place on diets deficient in copper, manganese, and zinc. There was a complete lack of vitamins. He did not attempt to remove the zinc from the diet but added the metal to a basal ration low in zinc. He found some favorable effects on the time of survival due to the addition of 25 parts per million of zinc.

Both the above studies are so complicated by a lack of the essential dietary principles that they cannot be in any way accepted as proving a favorable action of zinc. Thus it was that Hubbell and Mendel (9) attempted again to solve the question of the importance of zinc in a complete diet. They found, however, that it was practically impossible to free their casein and their source of water-soluble vitamins from it. These contained, as used, 4 and 14 parts of zinc per million respectively. Therefore they had to content themselves with feeding a zinc-low diet furnishing mice with about 0.005 mg. per day. Assuming mice to eat about 3 gm. of food daily, this would be a concentration of about 1.67 parts per million of zinc. They fed groups of controls the same ration with the addition of 0.02 and 0.04 mg. of zinc per day and also a control diet which was estimated to furnish 0.318 mg. of zinc per day. Great care was taken to exclude dust from the cages for that was found to be a source of zinc. As a result of this experiment they found that there was a slight retardation of growth when the animals were fed on a zinc-free diet and the addition of zinc caused a slight stimulation to growth, but it was not sufficient to make the diet equal to standard. No considerable differences were observed. The animals fed on the zinc-low diet showed a distinctly lower zinc content than those on the control diets. A decrease of from 0.021 to 0.016 mg. per gram for the females and from 0.019 to 0.012 for the males was noted. Those animals which received slight additions of zinc showed some increase in body zinc but the controls receiving 0.318 mg. per day had a smaller zinc content per gram of mouse than those receiving 0.224, 0.04 or 0.02 mg. per day. There did not seem to be any storage of zinc in the actively growing mouse. Hubbell and Mendel conclude that it is possible that the value of zinc lies not alone in the metal itself but it may be in some way associated in function with other metals present in small amounts. It is not unlikely, however, that there is some variation in growth with varying amounts of zinc and the metal is not merely an accidental factor in the nutrition of the mouse.

Thus it is that, in spite of the widespread occurrence of zinc in plant and animal organisms (10), no one has demonstrated conclusively any specific effect of this metal in animal nutrition. For this reason and as a part of an extended series of studies on mineral metabolism, we have studied the effect of practically zinc-free diets on the growth and health of rats.

#### EXPERIMENTAL WORK

The preliminary studies of foodstuffs were made by means of spectrography. The apparatus and main details of the method of its use have been described by Tourtellotte and Rask (11). The sample of material to be examined was charred in a low, wide silica dish on a hot plate, ignited in a muffle at 450°C. as measured by a pyrometer. This procedure was shown to be satisfactory by Thompson (12) and also recommended to the Association of Official Agricultural Chemists (13). When the sample did not yield a white ash after 4 or 5 hours, it was treated with a few cubic centimeters of redistilled nitric acid, evaporated to dryness and the heating continued. The extraction of the ash through filter paper was avoided. The white ash was washed into a small beaker and the silica dish rinsed with a few cubic centimeters of concentrated redistilled sulfuric acid which was added to the contents of the beaker. A little redistilled nitric acid was added to oxidize the iron and the solution evaporated to the appearance of white fumes. It was then allowed to cool, diluted to about 75 cc. with distilled water, and approximately tenth normal zinc-free sodium hydroxide was added until the solution was alkaline to phenolphthalein with one or two cubic centimeters in excess. This solution was electrolyzed in the cold with constant stirring using a platinum anode and two pure copper rods as the cathode. The rods were dipped about one centimeter into the solution and a current of about five hundredths of an ampere was passed through them for 3 hours. They were then rinsed with distilled water and dried in the air. They were stored in test tubes until ready for use in spectrography. The rods were used as electrodes for the arc or spark and the light from this source was photographed by a Hilger E-1 spectrograph.

Little difference was detected in the sensitivity of the detection of zinc whether the arc or spark was used to excite the spectrum. The spark was preferred since the lines did not fog over on the adjacently photographed spectrum.

The photographic plates were examined with a hand lens having a power of fourteen diameters. The lines examined for were 4811 Å, 4722 Å and 4680 Å. Twyman (14), in his "Wavelength Tables" reproduces de Gra-

mont's tables of the *raies ultimes* (lines of greatest sensitivity) in which the first two lines are given as the *raies ultimes* of zinc. In all work the spectrograph was set so that these lines were at minimum deviation.

The sensitivity of the method was tested by electroplating zinc from solutions of known strength and examining the spectra from these plates. The effect of ashing was ascertained by adding known amounts from 0.0001 mg. to 1.0 mg. of zinc to ten gram samples of sugar and then carrying out the procedure as described above. Amounts of zinc down to 0.001 mg. could be detected. This is not as sensitive as the results of Bayle and Amy (15) who detected  $10^{-8}$  gm. of zinc by a similar method.

Only the crudest quantitative estimations were attempted by this spectrographic method. Its main purpose was to furnish definite assurance that the substance observed was actually zinc. The line 4811 Å was found present faintly and by itself in the spectrograms from 0.001 mg. of zinc. In a ten gram sample, as usually taken, this would represent a concentration of  $10^{-7}$  of zinc. When the spectra of the ash of the diets showed the absence or only faint presence of this line they were called zinc-free.

In the instances where quantitative determinations were desired the turbidimetric method of Fairhall and Richardson (16) was employed. However, this method gave trouble in the cases where the whole bodies of large rats were analyzed. The calcium was precipitated as citrate in the first adjustment of the acidity and this carried through the subsequent precipitations of zinc to the final turbid solution where it produced an interference. In solutions having less calcium the method yielded good results.

Both the spectrograph and the turbidimetric method agreed that the concentration of zinc in the zinc-free diets was much less than 1 part per million.

The spectrograph showed traces of zinc to be present in the following food substances taken from the stock at hand: casein, dextrin, butter, wheat germ oil, raw starch, starch washed twice with two per cent hydrochloric acid, starch washed four times with 5 per cent hydrochloric acid, viosterol, and both 50 and 70 per cent alcoholic extracts of yeast. No zinc was found at any time in the salt mixtures. Thus it was found necessary to purify practically all the foods fed in this experiment.

An attempt was made to have the diet as simple as possible. In brief, the essentials were thought to be a suitable protein, carbohydrate, linoleic acid, salts, vitamins A, D and E, and the water-soluble vitamin B and G complex.

### *Protein*

Skim milk was electrodialed in a porous clay battery jar eleven inches high and four inches in diameter. A platinum anode surrounded by an alundum tube eleven inches long and one inch in outside diameter which was supported by corks on a long glass stirring rod was placed in the center of the above jar and constantly rotated by a motor. The jar was wrapped with copper fly screening to serve as a cathode and was set in a copper can through which a stream of tap water was passed during the electro dialysis. This apparatus would hold about a liter and a half of skim milk. A current of about 1.4 amperes was passed through the milk and after about two hours the casein would float as a coagulated mass leaving a yellowish liquid. The liquid was then strained off through a piece of muslin, the casein collected and stored in alcohol until sufficient amount was obtained. Since alcohol may contain zinc it is well to redistill all of that used for this purpose. Contamination of one batch of casein was thought due to the lack of this precaution. The casein was dried by spreading in flat pyrex dishes and passing over it a current of warm air. By making three or four runs a day about a gallon and a half of milk could be used and 200 grams of air dry casein produced.

The casein so prepared was spectrographically free from zinc.

### *Carbohydrate*

Zinc seemed to be intimately bound in the starch granules so that it was impossible to obtain zinc-free starch. For this reason sucrose was employed as the source of carbohydrate. Its effect in the diet had been investigated by Jackson (17) who reported little, if any, harmful effects in proportions as high as 80 per cent. Commercial sugar was found to have a low but variable zinc content. Merck's C.P. sucrose was found to have a zinc content of the order of  $10^{-7}$  part. This was used to make up the zinc-free diet.

### *Salts*

The stock salts 51 of this laboratory were found to be free from zinc when tested either as separate salts or as a mixture. Their composition is as follows:

	parts per hundred of food
Calcium carbonate	1.5
Potassium chloride	1.0
Sodium chloride	0.5

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Sodium bicarbonate	0.7
Magnesium oxide	0.2
Ferric citrate	0.5
Monopotassium phosphate	<u>1.7</u>
	6.1

Manganese occurred as an impurity in the above mixture.

Copper was added to the diets separately as described later.

Iodine solution was added to the drinking water once every week.

#### *Fats and Vitamins A and E*

Butter was chosen to provide these essentials. Simmonds, Becker, and McCollum (18) found diets containing 5 per cent of this to promote fair to excellent nutrition and fairly good reproduction. Similar diets containing 10 per cent of butter fat resulted in good nutrition and normal deliveries.

Butter fat contains appreciable amounts of zinc. This was removed by dissolving the butter fat in dichlorethylene, washing four times with approximately normal hydrochloric acid, twice with water and drying over sodium sulfate. The dichlorethylene was then distilled off in vacuo at about 55°C. Later the use of the solvent was discontinued for the butter could never be freed from a smell of it and it was thought to have some effect on the growth of the animals. The butter fat was washed while in a melted state instead of being dissolved. The first lot of butter lost its vitamin potency in 4 months even though kept in a refrigerator, so in later experiments only enough butter for 3 or 4 weeks' use was made up at a time.

#### *Vitamin D*

The ordinary viosterol was found to contain zinc; however, this was all removed by four washings with normal hydrochloric acid in the manner described for the treatment of butter.

#### *The Water-soluble Vitamins*

This group presented the greatest difficulty, and failure to make a satisfactory preparation of them has probably been at the bottom of the nutritional insufficiencies that occurred in the feeding experiments.

Attempts were made to free yeast extracts of zinc by electrolysis and by precipitation with hydrogen sulfide in the presence of copper acetate, but without success. An attempt to raise bakers' yeast in a synthetic medium lacking in zinc produced only a small growth of yeast which had no vitamin

potency. The lack of vitamin potency was not connected with the absence of zinc from the medium, for the addition of zinc to the same medium caused an increase in growth of yeast, but this yeast had no beneficial effect when fed to rats suffering from a vitamin B deficiency.

The source of vitamins B and G finally adopted was a modification of the preparation of Seidell (19). Dried yeast<sup>1</sup> was suspended in 50 per cent alcohol in the ratio of one liter of alcohol per 100 gm. of yeast. Alcohol was used since water extracts were found to be impossible to filter. This suspension was put into three-liter flasks and hydrogen sulfide run in until the suspension seemed saturated. The flasks were shaken frequently. They were then stoppered tightly and allowed to stand over night. The liquid was filtered off through coarse paper and treated with 37.5 gm. of Lloyd's reagent for each hundred grams of yeast taken. The suspension was stirred mechanically for one hour and then the liquid was filtered off through ashless paper and discarded. The activated Lloyd's reagent was sucked as dry as possible and, in later experiments, dried in a current of warm air, ground and fed in the diet. During the first feeding experiments the vitamins were still further extracted from the activated reagent by ice-cold 2 per cent sodium hydroxide which was quickly neutralized by hydrochloric acid. The resulting solution was evaporated on a boiling water bath in vacuo and the concentrated liquid poured over a measured amount of casein and dried in a current of warm air. This process added a considerable amount of sodium chloride to the diet, lowered the potency of the vitamins and was tedious. It was found later that the activated reagent was practically zinc-free in itself, so further extraction was discontinued. Copper sulfate solution calculated to produce ten parts per million of copper in the diet was added to the vitamin preparation before it was dried. Zinc dissolved in hydrochloric acid was added in the same manner when so desired.

#### THE ANIMALS AND THEIR CARE

The animals were obtained from a mixed colony of rats (*Mus norvegicus*). There were four strains,—white, yellow, brown, and black, which had been interbred for 19 years so that they are quite uniform in rate of growth and reproduction. They were started on the experimental diets a short time after being weaned, when their weights were between 35 and 50 gm. Five of them were kept in a cage and no bedding was supplied except when females had young. The female was put in a separate cage and strips of filter paper were then supplied for building a nest.

<sup>1</sup> Northwestern Yeast Co.

The cages for the zinc-free rats were made from iron hardware cloth of three meshes to the inch. This had not been galvanized and was spectrographically free from zinc. Large tinned cans, also zinc-free, were cut up and used for the frame work. The feed cups were tin plated sheet iron and were spectrographically zinc free.

All the cages had wire netting false bottoms to prevent the animals from eating their feces and thus taking in again any zinc excreted by that route. The wire bottoms had the further advantage of minimizing any contamination due to dust. For this same reason all the zinc-free cages had solid tops which prevented dust from settling in them. The control rats receiving zinc were kept in galvanized cages of approximately the same size as the zinc-free ones. These too had wire false bottoms.

The animals were supplied with food and water in abundance. The solid food materials were ground to a fine powder and thoroughly mixed. The butter fat was melted in warm water, the viosterol added to it, and incorporated into the mixture. The animals therefore could not select any one component of the diet.

The water used was the ordinary laboratory distilled water from a block tin still. No zinc was found in a hundred cubic centimeters of it.

#### *Diets*

##### Diet I.

Electrodialyzed casein	180 grams
Yeast grown on a zinc-low medium	50
Acid-washed butter fat	80
Salts 51	61
Sucrose, Merck's C.P.	629
	<u>1000</u>

Viosterol	15 drops
-----------	----------

Diet II was a control on Diet I and was of the same composition with the addition of 20 mg. of zinc in the form of its sulfate.

##### Diet V.

Electrodialyzed casein	180 grams
Acid-washed butter fat	50
Salts 51	61
Sucrose, Merck's C.P.	609
	<u>900</u>

Yeast extract equivalent to 100 gm. dried yeast	
Viosterol	15 drops

Diet VI was a control on Diet V and was of the same composition with the addition of 200 mg. of zinc carbonate which contains roughly 100 mg. of zinc.

Diet VII.

Electrodialyzed casein	180 grams
Acid-washed butter fat	50-80
Salts 51	61
Sucrose, Merck's C.P.	609-579
	<u>900</u>

Yeast extract equivalent to 100 gm. dried yeast.

Viosterol	15 drops
Copper as $\text{CuSO}_4$	10 mg.

Diet VIII was a control on Diet VI and was of the same composition with the addition of 100 mg. of zinc.

Diet IX was another control on Diet VII and was the same with the addition of 20 mg. of zinc.

Diet XI.

Electrodialyzed casein	180 grams
Acid-washed butter fat	80
Salts 51	61
Sucrose, Merck's C.P.	579
	<u>900</u>

Activated Lloyd's reagent equivalent to 150 gm. of dried yeast

Viosterol	15 drops
Copper as $\text{CuSO}_4$	10 mg.

Diet XII was a control on Diet XI and was of the same composition with the addition of 20 mg. of zinc. Later stock acid-washed casein was substituted for the electrodialyzed casein.

Diet XIII was the same as Diet XI with the addition of 100 mg. of zinc. Later stock casein was substituted as in Diet XII.

Diet XIV replaced Diet XI and was of the same composition except that stock acid-washed casein was used.

Diets I, V, and VII were found by the spectrograph to have no zinc or at times slight traces corresponding to amounts of the order of  $10^{-7}$  part. The casein used in preparing Diet XIV and later samples of Diets XII and XIII was found by analysis by the turbidimetric method to have a zinc content of 4.8 parts per million of zinc. Samples of Diet XIV showed a zinc content of 0.6 and 0.8 part per million.



## FEEDING EXPERIMENTS

In the first instance 5 rats were fed Diet I and 5 others of the same age were put as controls on the zinc-containing Diet II. The growth failed with all the symptoms of vitamin B deficiency in both groups. Three of the zinc-fed and one of the zinc-deficient animals died of these symptoms. The surviving zinc-deficient animals were killed for analysis while the controls were used to assay other vitamin preparations. No significant effect on the symptoms of vitamin deficiency was observed due to the presence or absence of zinc.

In the second experiment the source of water-soluble vitamins was improved by using the yeast concentrate described before. Diet V was fed and Diet VI containing roughly 100 parts per million of zinc was used as a control. Four animals of the same age were put on each diet. However, in this experiment the butter fat used had stood in the ice box from January to April. It still had a smell of dichlorethylene and had turned white. The animals fed on this developed a severe xerophthalmia and, though they had not begun to decline in weight, they were not considered worth carrying over the summer and so were killed for analysis.

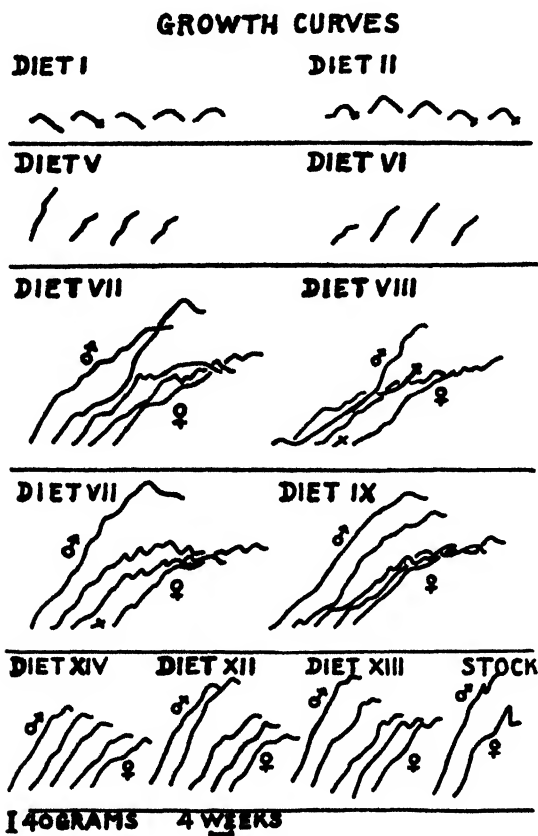
The butter fat had been cut to 5 per cent in this experiment, for the young rats on the previous diet had developed a very greasy condition of the fur. The amount of zinc in the control was raised to intensify any action which it might have on the growth of the rats.

No difference was observed in the rate of onset or the appearance of the symptoms of the xerophthalmia, so far as this condition had progressed before the animals were killed, between the zinc-deficient and the control zinc-fed animals.

A third set of animals was started on Diet VII with Diet VIII containing roughly a hundred parts per million serving as a control. At the start of the feeding period the weather was sultry and the rats obviously suffered. Thus the early growth curve is poor. After 12 weeks the butter fat was raised from 5 to 8 per cent and the sucrose cut down a corresponding amount. This had no noticeable effect on the growth but it was hoped that the added butter would supply more of the vitamin E.

Four weeks after the third group had been started on Diet VII the weather had moderated, so a fourth set was started on the same diet. However, the former control animals receiving Diet VIII, containing a hundred parts per million of zinc, were not growing well. It was thought possible that this amount was too much, so another control diet, Diet IX, was used to control this experiment. This diet contained roughly 20 parts per million of zinc.

The growth of the rats in this experiment was fairly satisfactory though it was not equal to the average for the stock rats in this colony. The almost complete absence of zinc (a concentration of the order of  $10^{-7}$  or less) had, if any, a slightly beneficial effect as compared with the controls. The number of rats was too small, however, to make any statistical study. Nine rats were raised to the peak of their growth curve on the zinc-free Diet VII. Five controls fed 20 parts per million of zinc in the same diet grew slightly



less well, and 4 controls fed 100 parts per million of zinc made the poorest growth.

The limiting factor in the previous diets was believed to be the water-soluble vitamins. Diet XI was planned to correct this by raising the amount of activated Lloyd's reagent in the diet to the equivalent of 15 per cent of dried yeast. Groups of 5 young rats were started on this diet, and Diets XII and XIII, which contained 20 and 100 parts per million of zinc re-

spectively. At the end of the first ten days it was found that the new batch of electrolyzed casein was contaminated with zinc. It was not possible to prepare more, so the stock acid-washed casein (washed 6 times with tap water and 7 times with 0.2 per cent acetic acid) was used to make up Diet XIV. This diet contained in the order of 1 part per million of zinc. Another group of 5 young rats was started on this diet while the rats which had been on Diet XI were transferred to a stock diet to serve as a control for the rate of growth of rats under the physical environment of the experiment.

This experiment was continued for a little over 13 weeks for the control rats and 12 weeks for the zinc-low rats on Diet XIV. The growth of this latter group was not as good as any of the controls particularly in the case of the males though comparison of the groups may be somewhat vitiated by the fact that those rats on Diet XIV were taken from a different litter.

The basal diet seemed to be lacking in some essential to optimal health for practically all rats on the Diets XII, XIII and XIV were affected by the period of sultry weather, which came at the end of the experiment, and lost weight. The animals on the stock diet were not affected to such an extent. Otherwise the growth curves of all the groups are quite similar.

### *Reproduction*

The reproduction on the experimental diets was very unsatisfactory; while each of the females on the stock diet had normal deliveries and one was allowed to wean its litter successfully, none of the females on the other diets had a normal number of young and none of the young survived more than a few days.

One of the zinc-free females had 2 young by a zinc-free male on Diet VII. These were the only young born on the zinc-free diets even though a stock male was later put with the females. The young were born alive but were very small, weighing together only 7 gm. The mother was solicitous, built a nest of paper and attempted to nurse the young, but they died the following night apparently from inability to suckle.

The zinc content of the young was determined. The amount was about one-quarter of that found in normal stock young.

No young were born to rats on the diets containing 20 parts per million of zinc while 3 females on diets containing 100 parts per million of zinc gave birth to a total of 8 young. None of these young survived more than a day.

At the termination of the feeding period for the rats on Diets XII, XIII and XIV the animals were autopsied. One of the females on the zinc-low Diet XIV had 4 small fetuses in the uterus. Among the group on Diet XII

containing 20 parts per million of zinc, one female had 4 and another 2 fetuses. Among those getting Diet XIII containing 100 parts per million of zinc one female had 10 small fetuses while another had 9. This would tend to indicate that fertilization of the egg takes place on these diets and the subsequent failure to reproduce is due to the death of the fetus in utero.

*Effect of the Zinc-free Diet on the Zinc Content of the Body*

At the start of the first feeding experiment 4 rats of the same age as the experimental animals were killed for analysis. Their bodies were slit open and the whole rat dried without washing out the intestines. When the rats on Diet I were almost at the point of death from vitamin deficiency the surviving 4 were killed and dried in the same manner. Thus we have a comparison between animals raised on a stock diet and similar animals carried 6 weeks further on a zinc-low diet.

On the termination of the experiment with Diets V and VI the rats

ANALYSES OF RATS BY FAIRHALL AND RICHARDSON'S METHOD

	Weight of rat	Total zinc	P.P.M. zinc
	grams	mg.	
Stock rats at beginning of experiment . .	59	1.20	20.0
	62	1.10	17.7
	62	1.00	16.1
	65	1.20	18.5
			av. 18.1
Rats on Diet I (zinc free). . . . .	37	0.20	5.4
	49	0.40	8.2
	35	0.25	7.1
	66	0.50	7.2
			av. 7.0
Rats on Diet VI (100 p.p.m. zinc). . . .	79	1.60	20.3
	105	1.60	15.2
	120	1.90	15.8
	92	0.70	7.6
			av. 14.7
Rats on Diet V (zinc free). . . . .	151	0.50	3.3
	109	0.45	4.1
	106	0.40	3.8
	94	0.70	7.4
			av. 4.7

## ANALYSES OF YOUNG

Diet of parents	No. of young	Total weight	Total zinc	P.P.M. zinc
		grams	mg.	
VII	2	7	0.04	5.7
VIII	4	19	0.38	20.0
Stock	3	18	0.37	20.5

were killed and the lower intestines washed out with distilled water before the bodies were dried for analysis. Here we have a comparison of rats fed a zinc-free diet compared with mates of the same age fed the same diet with the addition of 100 parts per million of zinc over a period of 5 weeks.

The zinc content of the zinc-fed controls and the stock rats was roughly the same. Animals on the zinc-free diet lost not only in the proportion but also in the actual amount of zinc present in the body.

## CONCLUSIONS

A diet has been prepared which supports growth in the rat and having a zinc content which is of the order of  $10^{-7}$ , or less, part.

Zinc is probably not an essential nutritional factor in the growth of the rat.

Normal reproduction was not obtained on the experimental diets or controls but conception and successful gestation took place when both parents had been raised on a zinc-free diet.

The young born to a female on such a diet had a greatly diminished zinc content as compared with young born to a female on a stock diet.

The zinc content of the rat's body is somewhat mobile and depends, to some extent, on the content in the diet.

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By

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Purdue University, Lafayette, Indiana*)

Received for Publication—July 15, 1932

SINCE calcium is one of the minerals which is given first consideration in planning an adequate diet, it seemed of interest to investigate the calcium utilization of one of the commonly used fresh thin green leafy vegetables, namely, leaf lettuce (*Lactuca sativa*).

Human adults have served as subjects in the studies on the calcium utilization of various forms of milk (1 to 8) and also in the studies on the calcium utilization of plant foods (1, 2, 3, 9, 10). In the study of the availability of the calcium of spinach, McLaughlin (3) found it to be a satisfactory source of calcium for seven adult women when spinach furnished 70 per cent of the total calcium intake. The average calcium intake for these subjects was 9.1 milligrams per kilogram of body weight. Six subjects were in positive calcium balance and one was in equilibrium. Rose (1) reported, in a calcium retention study on carrots with four adult women on an average intake of approximately 5.9 milligrams of calcium per kilogram of body weight, positive calcium balances in all subjects except one, who had found the carrot diet slightly less satisfactory for calcium retention on account of digestive disturbances. One subject had nearly the same calcium retention when approximately 55 per cent of the total calcium intake was furnished by the carrots as when milk furnished 70 per cent of the total calcium intake. The results of a study on five healthy women, reported by Pittman (9), in which navy beans furnished 80 to 85 per cent of the total calcium intake, seemed to indicate that 5.5 to 6.5 milligrams of calcium per kilogram of body weight were insufficient for calcium equilibrium. When almonds, as reported by Rose and MacLeod (2), furnished 73 to 86 per cent of the total calcium intake for nine adult women, the subjects, with two exceptions, were practically in calcium equilibrium. The daily calcium intake per kilogram of body weight ranged from 4.1 to 9.4 milligrams of calcium. Blatherwick and Long (10) in a study including a mixture of vegetables, some of which were consumed uncooked, others cooked, and still others canned, found positive calcium balances when the calcium

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intake of the two young women was approximately 9 and 13 milligrams per kilogram of body weight (calculated from data published). In their study this statement was made, "The commonly accepted values, as given in the different books by Sherman, were used in computing the calcium and phosphorus contents of the diets."

On a study of two adult dogs McClugage and Mendel (11) showed that the calcium of the vegetables, spinach and carrots, is not so well utilized as the calcium of milk. The calcium utilization of the dried spinach was very poor even on the intake as high as 30 milligrams of calcium per kilogram of body weight for dog B (calculated from data published). The same was true for Dog A when given 20 milligrams of calcium per kilogram of body weight per day. In the experiment in which dried carrots were used the calcium utilization was also poor for dog B when the calcium intake was 22 milligrams per kilogram of body weight per day. Dog A showed poor utilization when 15 milligrams of calcium per kilogram of body weight per day were furnished by the dried carrots. The investigators believed this might be due to the large amount of bulk in the diet, since the fecal mass increased 90 to 100 per cent when the vegetables were fed.

Sherman and Hawley (12) found the calcium balances of children, ranging from 5 to 13 years of age, less satisfactory when carrots and spinach replaced one-half of the milk as the source of the calcium in one series; and the same when carrots, spinach, and celery replaced one-half of the milk in another series.

#### EXPERIMENTAL PROCEDURE

Two healthy young women, one an assistant in Nutrition in the School of Home Economics and the other a graduate student in Nutrition, served as the subjects. They were comparatively similar in weight, namely, 52.3 and 50.2 kilograms. They were well within the correct range of weight for their height, had lived on an adequate diet previous to these studies, and lived healthy, active lives. During these studies the subjects were engaged in the same activities.

Before participating in the lettuce study both subjects ate a quantity of lettuce which would be necessary to meet the calculated requirement for calcium equilibrium and which would represent a high percentage of the total calcium. During this time the subjects abstained from other vegetables and fruits. It was found that the subjects experienced no difficulty in consuming the large amount of this leafy vegetable for three consecutive days during February, nor did they experience any digestive disturbance.

The studies for the utilization of the calcium extended over a period of 18 days during March and April. The 18 days were divided into two periods of 9 days each, one for the study of leaf lettuce and the other for the study of pasteurized milk. Each 9-day period consisted of a 3-day preliminary period followed by a 6-day experimental period. During the preliminary period the same weighed amounts and kinds of food were eaten as those of the experimental period.

The diets were planned to furnish an amount of calcium that would be close to the calculated average requirement for equilibrium (13) and to meet the protein and energy requirements of the subjects. In Table I is given the amount of food consumed daily and also the calcium content of each food according to chemical analysis. The two subjects ate similar amounts of food. During the leaf lettuce experimental period, Subjects I and II consumed 54 grams protein, 83 grams fat, and 263 grams carbohydrate which yielded about 2,000 calories as calculated from Rose's Tables (14). During the pasteurized milk period Subject I consumed 53 grams of protein, 81 grams of fat, and 269 grams of carbohydrate which yielded about 2,000 calories. Subject II ate a similar calculated amount, namely,

TABLE I  
DAILY FOOD INTAKE

Food	Leaf Lettuce Experimental Period				Pasteurized Milk Experimental Period			
	Subject I (52.3 kg.)		Subject II (50.2 kg.)		Subject I (52.3 kg.)		Subject II (50.2 kg.)	
	Wt.	Calcium	Wt.	Calcium	Wt.	Calcium	Wt.	Calcium
Lettuce, leaf, E.P. . . . .	gm. 624	gm. .493	gm. 595	gm. .482	gm. —	gm. —	gm. —	gm. —
Milk, whole pasteurized . . .	—	—	—	—	373	.451	369	.446
Apples, fresh, cored but un- pared . . . . .	—	—	—	—	400	.024	400	.024
Beef, lean round . . . . .	185	.011	185	.011	150	.009	150	.009
Crackers, soda . . . . .	75	.026	75	.026	75	.026	75	.026
Butter-fat . . . . .	60	—	60	—	45	—	48	—
Sodium chloride C.P.* . . . .	2	—	2	—	2	—	2	—
Sucrose . . . . .	190	—	190	—	139	—	130	—
Total . . . . .		.530		.519		.510		.505

\* Calcium content of the sodium chloride, c.p., was determined and found to be 0.00002 gm. per 5 gm.

53 grams of protein, 83 grams of fat, and 260 grams of carbohydrate. The diet for the study of the calcium retention of leaf lettuce consisted of the ordinary strain of Grand Rapids leaf lettuce, lean round beef, soda crackers, butter-fat, and sucrose. In the milk study the diet was the same except that pasteurized whole milk replaced the leaf lettuce, and apples were added. Distilled water was used *ad libitum* for drinking.

#### DISCUSSION OF THE EXPERIMENTAL LETTUCE

The leaf lettuce used in the study was the ordinary strain of Grand Rapids lettuce. There were two lots, one being planted a little earlier than the other. Each lot was grown under the same conditions at the Purdue University greenhouse. "The crop of lettuce used in the experiment was a part of the second crop which had been grown on the soil after it was sterilized. Before sterilization, a very heavy application of stable manure was spaded into the soil. There was no commercial fertilizer added to the soil."<sup>1</sup>

Enough mature lettuce for a 3-day period was gathered at one time. Each leaf was freed from adhering soil and moisture by use of a towel. Each day's supply for each subject was weighed, placed in a dampened cotton bag which was in turn placed in a heavy brown paper bag, and kept in a refrigerator to prevent wilting. At the same time a thousand gram sample was weighed and allowed to dry in the air in preparation for the calcium analysis.

Approximately one-third of a day's supply was eaten at each meal. It was washed in distilled water before consumption. The daily weighed amount of lettuce was measured by volume and was found to be about a peck. At meal time the lettuce was placed in a bowl on the table and one or two leaves were rolled together and eaten like celery. There was no difficulty in consuming this amount. The water intake, however, was decreased during the lettuce experimental period. It was noted by the subjects that the lettuce was of excellent quality, tasted sweet, and that it contained little cellulose as compared with head lettuce.

The calcium content of the lettuce is given in Table II. From the time that the first lettuce was gathered until the end of the experiment, the weather was cool enough to prevent the spoiling of the mature lettuce left in the soil for a long period.

The calcium from the leaf lettuce furnished 93.0 per cent of the total

<sup>1</sup> Information furnished by Professor E. C. Stair, Department of Horticulture, Purdue University.

TABLE II  
CALCIUM CONTENT OF LEAF LETTUCE

Date	Calcium
1932	per cent (Wet basis)
March 9	0.071
March 13	0.075
March 23	0.085*
March 25	0.073
March 28	0.082
March 31	0.079

\* 6 analyses, all others in triplicate.

calcium of the diet for one subject and 92.9 per cent of the total calcium for the other. The calcium from the milk furnished 88.4 per cent of the total calcium of the diet for one subject and 88.3 per cent for the other.

The milk, consumed as purchased, was obtained fresh each day from the Purdue University creamery.

A sufficient amount of food, with the exception of lettuce, milk, butter, and meat, was purchased at one time for both experiments. The meat was lean round of beef purchased from the local market in two lots. It was trimmed of fat, ground, and well mixed. Samples were taken for calcium analysis. A day's supply for each subject was weighed for the entire experimental period, wrapped in oil paper, and kept in cold storage. The meat was baked in casseroles in a hot oven from 10 to 20 minutes, according to the preference of the subject. It was eaten from the same container in which it was baked in order to avoid loss. The apples, of the winesap variety, chosen for flavor, were purchased from the local market and were kept in cold storage. To meet the caloric requirement, sugar was eaten as pure cane sugar made into a flavored paste. The daily supply of sucrose was evenly divided for the three meals. The protein of the diet was of high biological value and was consumed in the amount of at least one gram per kilogram of body weight. The torsion balance used for the weighing of the food consumed had its weights checked to the second decimal place with quantitative weights.

For the calcium determinations weighed samples of leaf lettuce of a 3-day gathering (previously dried in the air), pasteurized milk (a composite sample for every three days), cored apple, lean ground beef, and soda crackers were dried in an electric oven and then ashed in porcelain crucibles according to the official method (15) using an electric muffle furnace, kept between 400–450°C.

TABLE III  
CALCIUM BALANCE (SIX DAYS)

Subject	Experimental period	Calcium Intake		Calcium Output			Balance	Balance per day	Balance per kg. per day
		Total	Per kg. body weight per day	Urine	Feces	Total			
Subject I Weight 52.3 kg.	Lettuce, leaf	gm. 3.180	mg. 10.1	gm. 0.508	gm. 1.843	gm. 2.351	gm. +0.829	gm. +0.138	mg. +2.6
	Milk, whole pasteurized	3.060	9.8	0.850	2.119	2.969	+0.091	+0.015	+0.3
Subject II Weight 50.2 kg.	Lettuce, leaf	3.114	10.3	0.219	1.860	2.079	+1.035	+0.173	+3.4
	Milk, whole pasteurized	3.030	10.1	0.638	2.201	2.839	+0.191	+0.032	+0.6

Urine and feces of the 3-day preliminary period were not collected. The first collection of the excreta was made the first day of the experimental period. The feces were dried and ashed for calcium analysis.

Calcium of food, feces, and urine was determined by McCrudden's (16) method with the pH value adjusted according to Shohl and Pedley (17). The 0.01N and 0.05 N  $\text{KMnO}_4$  solutions were prepared and standardized according to Halverson and Bergeim (18). The potassium permanganate solution used in any series of calcium determinations was restandardized before the titrations for calcium were made. For titrations U. S. Bureau of Standard burettes were used. Redistilled water was used for the preparation of all solutions and for rinsing of all apparatus used in making the analyses. The chemicals used were secured from the Mallinckrodt Chemical Works. The modified calcium method was subjected to preliminary testing for the recovery of calcium from known solutions. Determinations of calcium were made in triplicate.

#### DISCUSSION AND RESULTS

The calcium balances as given in Table III show a daily average balance of +0.138 grams for Subject I and a daily average balance of +0.173 grams for Subject II on a diet in which ordinary leaf lettuce of the Grand Rapids strain was used; and the calcium balances show a daily average balance of +0.015 grams for Subject I and a daily average balance of +0.032 grams for Subject II on a diet in which pasteurized whole milk was used.

A summary of the calcium balance experiments performed at Purdue University is given in Table IV. Subject M. J. has served in six of the studies; subject C.D. in four of the studies; and subject R.J. in two studies. Comparing the calcium retention of the ordinary strain of Grand Rapids fresh leaf lettuce with that of the milk and cheese studies done under similar conditions at Purdue University, it is noted that the calcium retention of the leaf lettuce is high; namely, 2.6 and 3.4 milligrams per kilogram of body weight for subjects M.J. and C.D., respectively.

Was the higher calcium retention due to the hypothesis early stated by Hart, Steenbock, Hoppert, and Humphrey (19) in 1922 that the fresh green plant favored calcium assimilation?

#### CONCLUSION

The calcium of the fresh green leafy vegetable, lettuce, which furnished 93 per cent of the total calcium, was superior in its utilization to that of

TABLE IV  
SUMMARY OF CALCIUM BALANCE EXPERIMENTS ON THREE YOUNG WOMEN OF SIMILAR AGE,  
WEIGHT AND HEIGHT AT PURDUE UNIVERSITY

Date	Subject	Body weight	Calcium from food	Calcium Balance (Average Daily) per kilogram of body	
				Intake	Balance
Raw Centrifuged Milk (Diet Containing High Fat)					
Feb. 1929*	M. J.	kg. 50.3	per cent 93.3	mg. 9.8	mg. -0.8
		50.3	93.0	9.4	+1.0
Feb. 1929*	R. J.	50.6	93.3	9.8	+0.9
		50.6	93.0	9.4	-1.9
Raw Centrifuged Milk (Diet Containing Low Fat)					
Mar. 1929*	M. J.	50.3	94.6	9.6	-0.2
		50.3	94.6	9.6	+0.9
Mar. 1929*	R. J.	50.6	94.6	9.6	-3.0
		50.6	94.6	9.6	-1.2
American Cheddar Cheese					
Feb. 1931†	M. J.	51.3	86.0	10.2	+0.1
	C. D.	50.2	85.7	10.2	-0.8
Pasteurized Whole Milk					
Feb. 1931†	M. J.	51.3	87.7	9.4	-0.2
	C. D.	50.2	87.4	9.4	-0.6
April 1932‡	M. J.	52.3	88.4	9.8	+0.3
	C. D.	50.2	88.3	10.1	+0.6
Leaf Lettuce					
Mar. 1932‡	M. J.	52.3	93.0	10.1	+2.6
	C. D.	50.2	92.9	10.3	+3.4

\* Mallon, Jordan, Johnson (6).

† Mallon, Johnson, Darby (8).

‡ Present Study.

pasteurized whole milk when these foods were fed on an approximately equal calcium intake to two healthy young women.

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## BERYLLIUM "RICKETS"

By

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TO PRODUCE experimental rickets in rats it has been found necessary, by almost every worker hitherto, to feed diets having two definite faults, first a gross mineral imbalance between calcium and phosphorus, and second a complete or very nearly complete absence of vitamin D. Human rickets, on the other hand, occurs almost always on diets which are adequate and fairly well balanced as far as their *mineral* salts are concerned, but which are lacking in vitamin D. Since both rat rickets and human rickets are curable by the addition of vitamin D in small quantities to the otherwise rachitogenic diet, they are, very definitely, deficiency diseases.

In beryllium "rickets" however, as will be shown in this paper, we are concerned with an experimental condition which may be developed in animals fed a *normal* diet, containing normal amounts of calcium and phosphorus and adequate vitamin D but to which has been added a relatively small quantity of (basic) beryllium carbonate, a condition which shows many of the characteristics, both histological and chemical, of orthodox experimental rat rickets<sup>1</sup> and, if the quantity of beryllium in the diet is sufficient, shows them in a grossly exaggerated form. Though we have not explored all the possibilities in this direction it appears that the severity of the rickets can be graduated with considerable nicety by adding more or less of the beryllium salt to the normal diet.

In investigating the effect of certain activators and inhibitors on mammalian phosphatase one of us found, some two years ago, that  $\text{Be}^{++}$  like  $\text{Ca}^{++}$  was occasionally strongly activating to the enzyme. As is indicated

<sup>1</sup> By "experimental rat rickets" we mean the type of bone lesion produced by feeding rats on high Ca-low P diets, such as those of McCollum (3143), Steenbock (2965) or Osborne and Mendel, and not the type of change, usually considered to be rather osteoporotic than rachitic, produced by a high P-low Ca diet.

by the position of both elements in Group 2 of the periodic table, beryllium has many points of chemical resemblance to calcium; for present purposes it is only necessary to mention that, in addition to an insoluble hydroxide and carbonate, it forms phosphates which, like the corresponding phosphates of Ca, are very insoluble at neutrality and on the alkaline side of it, but which, unlike the phosphates of calcium, are also relatively insoluble even in fairly acid media. In view of this last property, it occurred to us that interesting results might follow the introduction of beryllium salts into the diet of experimental animals, particularly since a preliminary trial showed that, when mixed with either a normal or a rachitogenic diet, beryllium carbonate was well tolerated by rats.

We first found (1) that a very severe type of rickets followed when the McCollum or the Steenbock rachitogenic diet, modified by replacing the calcium carbonate by its equivalent of beryllium carbonate, was given to growing rats (21 to 24 days old when placed on the diet). More recently we have added the beryllium salt in varying amount to the normal stock diet (Bills') and have found that when quantities of the order of 0.25 per cent or more are added, the diet produces severe rickets in growing rats. With quantities of beryllium carbonate even as low as 0.125 per cent of the diet, definite rickets ensues in about three weeks time.

#### EXPERIMENTAL

(a) *Gross changes produced by beryllium carbonate.*—The rate of increase of growth of the rats was, in all the beryllium carbonate-containing diets, greatly diminished, the effect being as marked when the salt was added to the normal as to the modified Steenbock or McCollum diets. Animals soon showed much less activity than normal controls; after about a fortnight, with the larger quantities of beryllium, and somewhat longer with the lesser quantities, the gait became waddling and the back arched. After 24–28 days on the diet, the animals which were now in every case much under the normal weight for their age, were killed, and the bones and other tissues subjected to macroscopic, x-ray, histological, and chemical examination. The thorax showed typical rachitic deformities, with very marked swelling of the costochondral junctions. All the bones were very thin and fragile. The organs appeared to be normal; there was a characteristic color of most of the soft tissues in the animals which had received the greater percentages of beryllium, probably due to the anemia which was found to be present.

(b) *X-ray Examination.*—The x-ray photographs, particularly of those

animals which had had 1 per cent or more of the beryllium salt added to their (otherwise normal) diet, showed bones of a density considerably below that of the controls, with almost complete lack of calcification in the epiphyseal cartilage. Since the atomic weight of beryllium is so low (9.1) it is of course possible that even if Be salts had been deposited in the bones they would not have given an x-ray shadow, but as will be mentioned later, chemical examination of the bone ash failed to reveal any detectable quantity of Be. With the lower quantities of the salt (0.5 per cent) added to the normal diet, the appearance of the upper end of the tibiae was somewhat similar to that of rats with ordinary severe experimental (low P) rickets, but the heads of the femora were almost completely uncalcified in the former case. With still smaller quantities of beryllium carbonate (0.25 and 0.125 per cent) the "rickets," though quite definite, was rather less severe than that usually met with after feeding the Steenbock or McCollum rachitogenic diet for the same period.

(c) *Histological findings*.—For study it was decided to section the lower end of the femur and the adjoining portion of the tibia. The use of both bones had the advantage that owing to the slight difference in the time of appearance of the centres of ossification of the two bones, a comparison was possible of the effect of the nutritional disturbances on bones in slightly different stages of development.

The quantity of beryllium carbonate given varied in amounts from 3 per cent to 0.12 per cent of the diet. X-ray photographs indicated the most marked alteration from the normal in the deposit of bone salts in the epiphyseal region of the bones of rats which received the maximum dosage.

Sections were cut in the sagittal plane. Rats under fifty days old which received 0.12 per cent (Plate 1, Fig. 2) and 0.25 per cent (Plate 1, Fig. 3) of the beryllium salt, and rats seventy-six days old which received 2 per cent of the salt, showed a band of calcification extending wholly or partially across the bone in the provisional zone and also small deposits of bone salts dotted here and there in the osteoid tissue.

In many cases the trabeculae of the diaphysis, and also the cortex, indicated mineral deposition much below normal.

In all cases there was an increase of intercellular matrix of the epiphysis, especially in the proliferation zone. In a few instances there was an invagination of the cartilage (including the proliferative zone) into the secondary centre of ossification. There was also a marked increase marginally in the amount of cartilage of the head, often extending shaftwards over the margins of the columnar epiphyseal cartilage.

The epiphysis was greatly deepened, yet the columnar arrangement was usually well maintained. The proliferative zone of fusiform and flattened cells so well described by Dodds (2) was hardly altered except for the increase of intercolumnar matrix. This zone was from 20–25 cells deep both in normal and in beryllium-fed rats. The extraordinary variation observed was the presence of long strings of globular cartilage cells (Plate 1, Fig. 9B). Whereas these columns normally contain five or six, in beryllium "rickets" they extended to fifty, sixty or even eighty cells in length. With the exception of the distal fifteen or twenty the columnar arrangement was well maintained. Many cells in the deeper portion of the metaphysis became flattened and fusiform in shape. The cytoplasm of some of these stained deeply with hematoxylin and exhibited vacuolation, while the nucleus retained its affinity for eosin. On the other hand, at the junction of the shaft and the metaphysis these cells lost this affinity for hematoxylin, the cytoplasm being pink and the nucleus outlined in red with a granular content. Mitotic figures were numerous among these latter cells and frequently as many as six daughter cells could be seen still held within the parent capsule. In the tibia in animals showing the gravest disturbance of bone development, cartilage elements involved the whole of the central zone of the metaphysis and extended to the metaphyseal margins, both above and below. Marginally the advent of capillaries was associated with the conversion of the cartilage into osteoid tissue. This tissue was very irregular in outline with vascularity much more in evidence along its cartilaginous margin. Occasionally small groups of globular cartilage cells were incorporated within the osteoid tissue, bone salts being rarely present in this tissue.

The trabeculae of the diaphysis were usually narrow and less in evidence toward the centre, while at the margin they were numerous and regular.

Centrally the bone marrow extended in most cases almost to the level which had been occupied by the provisional zone of calcification at the time when beryllium was added to the diet. Usually small and occasionally large vacuoles were observed in this marrow. This vacuolation was well marked in rats which had received the Steenbock diet without added  $\text{CaCO}_3$ .

In Plate 1 will be found photographs of bone sections, illustrating certain of the points just mentioned, made from the tibiae and femora of rats with beryllium "rickets" of varying severity.

(d) *Chemical examination*.—The blood and samples of the other tissues, taken from all of the animals in a group (usually six), were pooled for

analyses. Precautions were taken to avoid, as far as possible, errors which might arise from rapid postmortem changes in the tissues which would affect, for example, the inorganic phosphate content of the blood, or the phosphoric ester content of the liver.

Typical chemical findings are summarized in Tables I and II.

TABLE I  
CHEMICAL FINDINGS IN BERYLLIUM "RICKETS"

Expt.	Diet	Bone ash per cent <sup>1</sup>	Inorg. P in plasma mg.	Phosphatase in			Remarks
				plasma units	bone units	kidney units	
916	Normal . . . . .	56	6.9	0.65	31	—	X-ray normal
	Steenbock 2965 with- out CaCO <sub>3</sub> . . . . .	48	5.5	0.86	37	—	X-ray no rickets density dimin- ished
	Steenbock 2965 no CaCO <sub>3</sub> but 2% BeCO <sub>3</sub> . . . . .	43	0.65	0.84	33	—	Rickets
	Ditto 2 drops viosterol daily . . . . .	43	0.84	0.48	21	—	Rickets
	Ditto ultraviolet light daily . . . . .	41	0.67	0.64	34	—	Rickets
	Ditto 2 drops C.l.o. daily . . . . .	42	0.65	0.64	24	—	Rickets
926	Normal diet . . . . .	59	8.6	—	23	49	X-ray normal Serum Ca. 9.85
	Steenbock 2965 no Ca- CO <sub>3</sub> but 1% BeCO <sub>3</sub> . . . . .	42	1.3	—	24	21	Rickets Serum Ca. 10.0
	Steenbock 2965 no Ca- CO <sub>3</sub> but 1% BeCO <sub>3</sub> . . . . .	44	1.2	—	31	12	Rickets, not very typical

<sup>1</sup> Percentage of dry, fat-free wt.

## DISCUSSION

One of the most striking features of experimental rickets and of the majority of cases of human rickets, is that the inorganic phosphorus content of the blood or plasma is low. In experimental rickets in rats, if the diet be properly planned, in spite of the low quantity of phosphorus ingested, and although there is defective deposition of both Ca and P in the growing bones, the growth of the soft tissues and the increase in total weight of the animal occurs at only a little below the normal rate. This points to fairly

TABLE II  
CHEMICAL FINDINGS IN BERYLLIUM "RICKETS"

Expt.	Diet	Bone ash percent	Inorg. P. in plasma mg.	Phosphatase in			Liver per 100 gms.			Remarks
				plasma units	bone units	kidney units	inorg. P mg.	acid-sol. P mg.	ester P mg.	
17	Normal.....	—	5.1	0.66	28	46	42	126	84	X-ray normal
	Normal+0.5% BeCO <sub>3</sub> .....	—	2.7	0.56	26	33	43	90	47	Rickets
	Normal+1.0% BeCO <sub>3</sub> .....	—	2.2	0.47	34	23	44	106	62	Rickets
	Normal+2.0% BeCO <sub>3</sub> .....	—	1.5	0.54	29	13	45	96	51	Rickets
154	Normal.....	57	7.9	—	—	36	49	134	83	Normal
	Normal+0.5% BeCO <sub>3</sub> <sup>1</sup> .....	35	0.78	—	—	20	52	99	47	X-ray rickets like
	Normal+1. % BeCO <sub>3</sub> .....	29	0.47	—	—	17	51	107	56	Steenbock
	Normal+2. % BeCO <sub>3</sub> .....	31	0.31	—	—	11	52	89	37	Severe rickets
	Normal+2. % BeCO <sub>3</sub> +cod liver oil..	32	0.27	—	—	12	51	91	40	Severe rickets
	Normal+2. % BeCO <sub>3</sub> +viosterol .....	28	0.25	—	—	12	50	94	44	Severe rickets
166	Normal.....	56	7.0	0.50	—	36	42	132	90	Normal
	Normal+0.125% BeCO <sub>3</sub> .....	41	3.0	0.42	—	37	43	100	57	Rickets
	Normal+0.25% BeCO <sub>3</sub> .....	38	2.9	0.41	—	35	43	105	62	Rickets
	Steenbock 2965.....	31	2.5	0.67	—	—	47	88	41	Severe rickets
	Steenbock 2965+cod liver oil.....	42	2.7	0.79	—	—	51	99	48	X-ray normal

<sup>1</sup> The serum calcium determined in two groups only, appeared to be normal.

efficient uptake from the intestine of the ingested phosphate, of which a considerable quantity is required for the growth of the soft tissues, particularly muscle and liver. It is to be remarked that in case of phosphate shortage in the diet, these tissues, in absence of vitamin D, appear to have first claim on the available phosphate supply, the demands of the bones remaining unsatisfied.

From Table II it will be observed that in many cases of beryllium rickets the inorganic phosphate content of the plasma is reduced to extremely low values, which are, as far as we can discover, the lowest plasma phosphate figures ever recorded in the literature. In some of the beryllium groups inorganic phosphate almost disappears from the plasma, and can only be estimated by the device of adding a small quantity of phosphate to the protein-free filtrate before estimation and subtracting the added quantity afterward. There is evidently a very acute shortage of this radical in the blood, which it seems legitimate to correlate on the one hand with the very poor growth and complete lack of calcification, and on the other with the extreme insolubility of beryllium phosphate at intestinal pH, or even at the pH of the gastric contents. Any phosphate going into solution in the fluids of the gut, or liberated by enzymic hydrolysis of phosphoric esters, will be immediately precipitated by the beryllium ions resulting from the solution of the basic carbonate in the gastric juice, and will thus be rendered unavailable for absorption through the gut wall. This extreme insolubility of beryllium phosphate even in acid range of pH is indicated by the following experiment. To a number of tubes containing 5 cc. phthalate buffers at pH's ranging from 2.2 to 5.4 was added 1 cc. of M/10  $\text{BeCl}_2$  solution, adjusted to  $\text{pH}_4$ . To a similar series of tubes was added the same quantity of M/10  $\text{CaCl}_2$  also adjusted to  $\text{pH}_4$ . Then to all the tubes was added 0.5 cc. of approximately M/10 potassium phosphate adjusted with dilute NaOH to the same pH as the buffers already in the tubes.

In the calcium tubes, a slight precipitate appeared at pH 5.4 but none at any lower pH. In the beryllium tubes, there was a large precipitate in the pH 5.4 tubes, and a precipitate in slightly diminishing quantities down to pH 2.6. The tube at pH 2.2 remained, however, quite clear. On filtering and carrying out phosphorus determinations on the filtrate, the following quantities of inorganic P were found

pH of tube:	below 1	2.2	2.6	3.0	3.4	3.8	4.2	4.6	5.0	5.4
mg. P per cc. of filtrate	1.64	1.64	1.36	1.03	0.86	0.51	0.38	0.26	0.20	0.14



Thus while under the above conditions there is no precipitate whatever of calcium phosphate at a pH of 5.0 or less, at this pH, and at pH's considerably more acid down to below 4, by far the greater part of the inorganic phosphate is precipitated by beryllium. Had the beryllium been present in excess, even larger portions of the phosphate would have been taken out of solution.

It has been found that at neutrality the precipitation of inorganic phosphate by slight excess of beryllium chloride is quantitative.

The amount of basic beryllium carbonate required to be added to a normal diet to bring about this acute bone dystrophy is only 0.5 per cent. Less than this amount (0.25 per cent and even 0.125 per cent) will produce definite rachitic changes (see Plate 1, Figs. 2 and 3). The normal stock diet we have used contains Ca 0.40 per cent and P 0.42 per cent, varying a little with the quality of the casein used. The basic carbonate employed by us contains 68 per cent  $\text{BeCO}_3$  and 32 per cent  $\text{BeO}$ , or about 20 per cent of Be. To combine with the whole of the phosphorus of the diet to form  $\text{BeHPO}_4$  would therefore require the addition to the diet of some 0.6 per cent, or to form  $\text{Be}(\text{H}_2\text{PO}_4)_2$  the addition to the diet of 0.3 per cent, of the basic carbonate. In accordance with this calculation it is found that in the bones of young animals which have been fed diets containing 1.0 per cent or above of the carbonate there is absolutely no evidence of calcification (x-ray) or phosphorification (von Kossa), with 0.5 per cent of the beryllium there is practically no calcification, and only with 0.25 per cent or less is any new bone salt laid down during the period of beryllium feeding. Even with 0.125 per cent of the carbonate added to the normal diet the inorganic phosphate concentration of the plasma is reduced, after three weeks feeding, to 2.4 mgm. P per 100 cc. below the usual rachitic level on the Steenbock or McCollum rachitogenic diet.

This question of the effect on the bones of the addition to a normal diet of very small quantities of beryllium salts is being investigated further, and it is not impossible that it may be due to other properties of beryllium than its power to precipitate phosphates quantitatively from slightly acid solutions.

Three other points of some interest arise out of the data presented in Table II. The first is that despite the astonishingly low plasma inorganic phosphate, the inorganic phosphate of the liver in beryllium rickets remains at its normal level; the second is that the phosphoric ester (acid-soluble organic phosphorus) of the liver is definitely diminished in this experimental condition (this is also the case in simple experimental rickets

in rats)<sup>2</sup>; the third is the progressive diminution in the phosphatase of the kidney tissue with the increasing beryllium carbonate in the diet. This last effect is not a necessary sequel to the rachitic condition, since it has been found (3) that even in severe "Steenbock" or "McCollum" rickets the phosphatase of the kidney is only slightly diminished. How this large effect is produced in beryllium rickets is quite unknown.

Of the influence on nutrition of the metals belonging to Group 2 in the periodic system which are not toxic on ingestion, that of strontium was investigated some years ago by König (4), by Stoelzner (5) and by Lehnerdt (6) and the results have been confirmed and extended by Shipley, Park, McCollum, and Kinney (7). All agree that strontium will not replace calcium in the diet, but if  $\text{Sr}_3(\text{PO}_4)_2$  is fed to puppies or young rabbits or rats it produces a type of bone dystrophy characterized by an excessive production of osteoid and by the presence of considerable quantities of Sr in the bones. Shipley and collaborators found also that the administration of cod liver oil in antirachitic doses does not prevent the onset of this type of "rickets" (termed by Lehnerdt "strontium sclerosis").

While these experiments with Sr are not strictly comparable with our own with Be, they have in common the finding that strontium "sclerosis" and beryllium "rickets" (at least when produced by 2 per cent beryllium carbonate in the "normal" diet) are both quite unamenable to treatment with cod liver oil. In addition, beryllium "rickets" is neither prevented nor cured by liberal doses of irradiated ergosterol or direct irradiation of the experimental animals. It was noticed, however, that both the cod liver oil and the irradiated ergosterol markedly increased the food intake of the treated animals. On the whole, in spite of the fact that growth was greatly interfered with, even on a 0.12 per cent beryllium carbonate diet, the food intake was good, and other things being normal, the amount eaten should have given good growth. The rats appeared not to suffer from intestinal disorder, and the feces were usually of normal consistency.

Semi-quantitative tests using (a) a sodium peroxide method of C. L. Parsons, described by Mellor (8); (b) a simple method founded on the relative insolubility of beryllium and the relative solubility of calcium and magnesium phosphates at pH 5.4, indicates that there is not, in any of the specimens of bone ash derived from the rats fed on diets containing beryllium, more than one part of Be to 250 parts of Ca. Actually there is probably considerably less than this. Thus there is no storage of Be in the bones on a Be-rich diet.

<sup>2</sup> To be published.

The results of these experiments appear to us to be consonant with the view that experimental rickets is primarily due to the deficient absorption of phosphate from the intestine (which view has received strong support recently from the work of Heymann (9)), and that the severity of the rickets depends on the extent of this deficiency.

Further work on the nutritional effects of beryllium salts is in progress.

#### SUMMARY

By replacing the calcium carbonate in Steenbock's rachitogenic diet 2965 with an equivalent amount of beryllium carbonate, or by adding small quantities of this substance to the *normal stock diet* (Bills'), bone lesions which have certain similarities to rickets may readily be produced in young rats, the severity of the lesions bearing an approximate relationship to the amount of the beryllium salt in the diet. The percentage of ash in the bones is much diminished. Both x-ray photographs and histological sections reveal almost complete failure of mineral deposition in the metaphysis, even immediately proximal to the epiphyseal disc, and reduced amounts of mineral salts in the trabeculae and cortex of the tibia. Other long bones are similarly affected. The depth of the metaphysis is comparable to that exhibited in severe Steenbock rickets. The maintenance of the columnar arrangement of the cartilage cells in the metaphysis is distinctive of beryllium "rickets" in the rat.

The inorganic phosphorus content of the blood plasma is very much reduced, and the acid-soluble phosphoric esters of the liver are diminished in quantity. The phosphatase content of the kidney is markedly lowered. Beryllium does not appear to be deposited in appreciable quantities in the bones. This type of bone lesion is not preventable by cod liver oil nor by irradiated ergosterol administration, nor is it amenable to the anti-rachitic influence of ultraviolet light.

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#### Plate 1

1. Von Kossa, Normal.
2. Von Kossa, Beryllium "rickets" 0.12 per cent beryllium in normal diet.
3. Von Kossa, Beryllium "rickets" 0.25 per cent beryllium in normal diet.
4. Von Kossa, Steenbock rickets (2965).
5. Von Kossa, High P "rickets."
6. Mallory, Normal.
7. Mallory, Beryllium "rickets" (3 per cent Be to Steenbock diet).
8. Mallory, Beryllium "rickets" (0.12 per cent Be to normal diet).
- 9A. Mallory, Rickets on Steenbock diet (2965).
- 9B. Rickets on Steenbock diet (2965) but having 2 per cent Beryllium carbonate in place of 3 per cent Calcium carbonate (showing relative regularity of cartilage proliferation in latter type of rickets).



1.



2.



3.



7.



3.



8.



4.



5.



9A.



9B.

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## STUDIES ON THE RELATION OF DIET TO GOITER\*

### I. A DIETARY TECHNIC FOR THE STUDY OF GOITER IN THE RAT†

By

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THE work of Remington and associates (1, 2) on iodine in human environment in South Carolina, as compared with that of McClendon (3) for states of the Northwest, coupled with the data collected by Olesen (4) as to incidence of thyroid enlargement in various parts of the United States, has aided in substantiating the correlation between an adequate iodine supply and the normal development of the thyroid gland, previously indicated by work in Europe and New Zealand. The examination of 17,600 school children of South Carolina, with a goiter incidence of only 3.8 per cent, as reported by Hayne (5) has added further weight to the contention that goiter is rare in this area, and that its scarcity can be correlated with an adequate supply of iodine.

The generally accepted theory that endemic goiter is due to an iodine lack rests on such chemical and clinical observations as the above, rather than on direct experimental evidence, which up to this time has been very meager, and for several reasons. First, methods for the reasonably accurate estimation of the minute amounts of iodine involved have not until recently been perfected, and second, a dependable dietary technic for goiter production in an experimental animal has not been devised. For these reasons our attention was first directed toward the development of a dietary technic which could be depended upon to produce marked thyroid enlargement and other symptoms referable to simple goiter in the rat so that later we might be able to carry out various studies on goiter.

A review of the literature revealed that several investigators (6-14) have carried out goiter studies using the rat as the experimental animal. A study

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of the various diets used by these workers to produce goiter indicated to us that they were not suitable, for several reasons. First of all, in some cases the production of goiter was brought about by means of rations which were deficient in one or more of the recognized dietary essentials other than iodine, while in certain instances the production of goiter was frequently accompanied by various types of infections which often resulted in the death of the experimental animals. Some workers were able to produce thyroid enlargement in some rats but not in others, while other investigators were able to produce only slight glandular enlargement, due presumably to the fact that the diet was only slightly deficient in iodine. With a few exceptions, iodine estimations on the diets were not carried out, thereby making correlations between the iodine intake, the iodine concentration in the thyroid, and the goitrogenic activity of the diet impossible. In the few instances where estimations of the iodine content of the diets were made, the values were obtained by analytical methods which are open to criticism. Some workers have concluded from their experiments that a lack or deficiency of iodine is not the cause of goiter, but such a contention has not been supported by a determination of the iodine content of the diets employed. A further study of the literature revealed the important fact that no investigator has apparently been able to bring about consistently a marked thyroid enlargement in the rat.

Since in our contemplated goiter studies, we were anxious to employ a goitrogenic diet of known iodine content which could be relied upon to produce consistently a marked thyroid enlargement in a short period of time, it was evident from the above critical study of the literature that we would be forced to devise such a suitable dietary technic ourselves.

In the adoption of a diet low in iodine for the study of goiter, we were guided principally by the findings of Krauss and Monroe (11) who discovered that the well-known rickets-producing ration of Steenbock and Black (15) produced, simultaneously with the rickets, enlarged thyroid glands in the rat. This diet has the following composition: yellow corn, 76 parts; wheat gluten, 20 parts; calcium carbonate, 3.0 parts; and sodium chloride, 1.0 part. These investigators, however, did not make a detailed study of the influence of this ration on the thyroid gland. Histological studies showing the effect of the goitrogenic ration on the structure of the thyroid gland were not made. Since the iodine content of the goitrogenic ration was not determined, Krauss and Monroe were unable to correlate the iodine content of this diet with the iodine content of the thyroid and in turn with the extent of thyroid enlargement. This information was

particularly desirable for our later studies on the determination of the minimum iodine requirement of the rat. These investigators also failed to ascertain conclusively whether the presence or absence of rickets and hence the presence or absence of the antirachitic vitamin D influenced the goitrogenic capacity of the diet. Both Mellanby (16) and Murray (17) have reported the occurrence of goiter along with rickets in the dog. Further, Krauss and Monroe report the average thyroid weight of only four normal stock rats. Accordingly, sufficient data to establish the normal thyroid weight of the rat were not available for comparison in calculating the extent of thyroid enlargement in the goitrous animals. With the above considerations in mind, we have made an intensive study of the influence of the Steenbock diet on the thyroid gland of the rat, and, as a result, have developed a dietary technic suitable for the study of goiter.

#### EXPERIMENTAL

The rats used in these studies were bred in our laboratory on an adequate stock ration which has been used in the production of many healthy generations of young for this and other experimental work. The composition of this stock diet is as follows: whole wheat (ground), 59.8 parts; whole milk powder,<sup>1</sup> 29.9 parts; Swift's meat scrap (55 per cent protein), 9.1 parts, and sodium chloride, 1.2 parts. In most of the studies, ten animals were placed on each diet so as better to control the factor of biological variation as well as to yield sufficient thyroid material to facilitate an accurate determination of the average iodine content of the glands in each group. The iodine content of all diets and food materials was determined by the methods employed in this laboratory (18).

At the end of the experimental feeding period, the rats were killed by means of chloroform. The thyroids were then removed and weighed immediately. In order to make fair comparisons of the effects of different diets on the weight of the thyroid, calculation of the fresh weight of the gland in mg. per 100 grams body weight was made. Next, the glands of all rats on the same diet were pooled and dried to constant weight at 80°. The iodine content of the dried composited glands was then determined by a modification of the micro-McClendon method (19). In some experiments, representative glands were saved for histological study.

In preliminary experiments, using 82 rats, we were able to confirm the findings of Krauss and Monroe (11) and of Clausen (20) relative to the marked thyroid enlargement produced by the Steenbock ration. The

<sup>1</sup> Klim.



following additional facts were also ascertained in these preliminary experiments:

(a) Iodine (4 to 5 $\gamma$  daily<sup>a</sup> as KI) reduces thyroid enlargement produced by the Steenbock diet, increasing simultaneously the iodine content of the gland.

(b) Goitrous glands were found to have a dark red color while normal glands were pale pink.

(c) Goitrous glands were low in iodine and in dry matter content.

(d) Rats bred on a high iodine stock ration were able to store iodine which subsequently retarded marked glandular enlargement when the animals were placed on the goitrogenic ration.

(e) Goiter develops in the presence or in the absence of rickets. A similar finding has been reported recently by Thompson (21).

(f) Although it was assumed (22-24) that the yellow corn of the ration furnished an adequate supply of vitamin A, the further addition of carotene to the diet did not influence glandular enlargement. McCarrison (10, 25) and Drennan, Malcolm and Cox (13) have made studies on avitaminosis A as a contributory cause of goiter.

(g) Anemia is not a concurrent manifestation of goiter. Anemia sometimes accompanies goiter in the human (26,27). However, ten goitrous rats showed normal hemoglobin values ranging from 14.5-17.1 gm. per 100 cc. of blood. The iron and copper content of the diet was shown by analysis to be adequate (50).

Based on our findings in the preliminary experiments, we adopted a modification of the Steenbock rachitic diet to be used as a standard goitrogenic diet in all subsequent experiments. To each 100 gm. of the Steenbock ration, we added 0.2 gm. of irradiated yeast<sup>b</sup> having a vitamin D potency fifteen times that of standard cod liver oil. We shall refer to this modified goiter-producing diet as Diet GP. Since we had found that a marked thyroid enlargement took place on Diet GP in the short period of 35 days, we decided to use this time interval as the length of the experimental period in subsequent experiments all of which were of the preventive type. Various batches of Diet GP were found to be low in iodine and to have an average iodine content of 15  $\gamma$  per kilo.

With the aid of the goitrogenic Diet GP, we have begun the study of

<sup>a</sup> The Greek letter *gamma* is used for 0.001 milligram.

<sup>b</sup> Cod liver oil could not be used as a source of vitamin D because of its high iodine content. The irradiated yeast was furnished us through the courtesy of the Fleischmann Laboratories, New York City.

many problems involving goiter. In the course of these experiments, it was deemed necessary to compare the gross and microscopic appearance, weight, iodine content, dry matter content, etc., of the thyroids of rats fed Diet GP with similar data obtained for animals fed either the stock ration or the goitrogenic diet to which iodine was added (Diet GPI<sup>4</sup>). With this information, we have been able to establish data for the *normal* thyroid from which the extent of glandular enlargement, loss in iodine content, change in the color, etc. resulting from the goiter-producing Diet GP were ascertained. Data on the three above mentioned diets have been collected from five series of experiments and are shown in Table I. Table II gives the average values for each diet shown in Table I and contains in addition data for the thyroids of stock rats at the start of the experimental period. The values given in Table II represent the averages of 1.—20 stock rats at the start of the experimental period, 2.—39 rats on the stock diet, 3.—193 rats on Diet GP and 4.—24 rats on Diet GPI.

A study of Table II reveals many striking facts which can best be brought out when discussed under various headings.

(a) *Appearance of the thyroid gland.*—Rats receiving Diet GP yielded enlarged thyroids, dark red in color, with considerable engorgement and distension of the blood vessels leading to the gland. In contrast to this finding, glands of rats on Diet GPI were pale pink in color with no engorgement of the blood vessels leading to the gland, while rats fed the stock ration yielded similar results except that the gland tended to be red rather than pink in color, indicating possibly that the stock ration contains an amount of iodine slightly below the requirement of the rat.

(b) *Histological findings.*—Marked hyperplasia together with little or no iodine-containing colloid was obtained on Diet GP. Thompson (21) also reports a similar finding using the same ration. On the other hand, Diet GPI yielded glands with a normal histological appearance containing considerable colloid, while the stock ration yielded glands that were nearly normal and contained less colloid than the glands of rats fed Diet GPI. Figure I shows the histological appearance of three representative rats (litter mates) fed the three rations. A report<sup>5</sup> on the histological findings in the thyroids of these rats is given below:

<sup>4</sup> The iodine was carefully incorporated in the ration by evaporating a solution of KI on a small portion of the ration. This material was then thoroughly mixed with the remainder of the diet.

<sup>5</sup> We are indebted to Dr. Kenneth M. Lynch, head of the Department of Pathology, Medical College of the State of South Carolina, for the preparation and interpretation of the histological sections of the rat thyroids.

TABLE I  
SHOWING THE PRODUCTION OF GOITER IN THE RAT AND ITS PREVENTION BY THE ADMINISTRATION OF IODINE  
(All rats started at approximately 60 gm. body weight\* and fed experimental diets for 35 days)

Series No.	Iodine content of diet	Iodine intake		No. of rats	Initial and final body wts.	Dry matter in thyroid	Average thyroid weight				Iodine conc. in gland		Total iodine in each gland	
		Av. daily	Av. total				Fresh		Dry		Dry basis	Fresh basis	Actual	per 100 gm. body wt.
							Actual	per 100 gm. body wt.	Actual	per 100 gm. body wt.				
	γ/kilo	γ	γ		gm.	%	mg.	mg.	mg.	mg.	%	%	γ	γ
Goitrogenic Diet—GP														
I	15	.13	4.6	16	60-90	19.5	69.1 (30.3-109.0)	78.1±2.9 (34.1-126.0)	13.5	15.2	.0064	.0013	0.86	0.97
II	17	.14	4.9	45	62-109	18.5	50.1 (25.8-88.6)	46.1±1.2 (24.1-72.7)	9.3	8.5	.0110	.0020	1.02	0.94
III	14	.13	4.6	29	60-102	19.7	42.6 (24.4-78.7)	41.8±1.9 (25.0-82.0)	8.4	8.2	.0102	.0020	0.86	0.84
IV	14	.14	4.9	53	63-112	18.6	69.3 (35.5-117.1)	62.1±3.2 (34.7-102.8)	12.9	11.6	.0060	.0011	0.77	0.70
V	15	.14	4.9	50	60-123	19.4	53.6 (32.6-93.6)	43.5±2.3 (26.1-63.5)	10.4	8.4	.0076	.0015	0.79	0.64

Goitrogenic Diet Containing Added Iodine†—GPI

II	Iodized H <sub>2</sub> O	†	—	4	61-112	26.5	13.1 (11.3-15.8)	11.8±.16 (11.2-12.3)	3.5	3.1	.2761	.0732	9.66	8.53
III	400	3.72	130.2	10	62-106	28.0	13.1 (10.2-17.4)	12.4±.25 (10.5-13.5)	3.7	3.5	.3038	.0851	11.16	10.40
IV	400	3.72	130.2	10	64-115	28.3	14.9 (11.4-17.7)	13.1±.36 (10.5-16.0)	4.2	3.7	.2275	.0644	9.60	8.42

Stock Diet

II	60	0.81	28.4	19	61-193	24.0	22.9 (17.4-33.2)	11.9±.28 (9.0-15.4)	5.5	2.9	.0666	.0160	3.66	1.93
III	47	0.51	17.9	10	60-160	21.2	23.4 (15.9-36.0)	14.5±.53 (10.9-18.6)	5.0	3.2	.0518	.0110	2.59	1.63
IV	72	0.82	28.7	10	58-169	24.0	22.3 (17.4-35.4)	13.3±.44 (9.8-16.3)	5.4	3.2	.0691	.0166	3.50	2.21

\* Average age = 27 days.

† As KI.

‡ Approximately 4 γ daily intake of iodine per rat in the form of iodized H<sub>2</sub>O containing KI.

TABLE II  
A SUMMARY OF DATA SHOWING THE PRODUCTION OF GOITER IN THE RAT AND ITS PREVENTION BY THE ADMINISTRATION OF IODINE  
(Rats started at approximately 60 gm. body weight\* and fed experimental diets for 35 days)

Diet	Iodine content of diet	Iodine Intake		No. of rats	Initial and final body wts.	Dry matter in thyroid	Average thyroid weight				Iodine conc. in gland		Total iodine in each gland	
		Av. daily	Av. total				Fresh		Dry		Dry basis	Fresh basis	Actual	per 100 gm body wt.
							Actual	per 100 gm. body wt.	Actual	per 100 gm. body wt.				
	γ/kilo.	γ†	γ		gm.	%	mg.	mg.	mg.	mg.	%	%	γ	γ
Stock	47-72	—	—	20	62	25.6	10.2 (8.9-13.8)	16.4 ± .32 (14.1-21.2)	2.6	4.2	.0428	.0110	1.11	1.80
Stock	47-72	0.74	25.9	39	60-178	23.3	22.9 (15.9-36.0)	12.9 ± .25 (9.0-18.6)	5.3	3.0	.0634	.0147	3.36	1.90
GP	15	0.14	4.9	193	62-109	19.0	58.0 (24.4-117.1)	53.2 ± .92 (24.1-126.0)	10.8	9.9	.0083	.0016	0.88	0.82
GPI†	400	3.72	130.2	24	63-111	28.0	13.8 (10.2-17.7)	12.6 ± .19 (10.5-16.0)	3.9	3.5 <small>mean ± S.E.</small>	.2671	.0745	10.28	9.26

\* Average age = 27 days.

† 1 mg. = 1000 γ.

‡ Containing added iodine as KI.

*Rat 551. Diet GP.*—Blood vessels engorged. No smooth colloid. Lumen of acini small. Content of acini stringy and granular. Lining cells large, with granular reticulated cytoplasm. Nuclei large and vesicular. Some intra-acinar papillary growth. Apparently a very active gland, with little colloid accumulation.

*Rat 553. Diet GPI.*—Gland normal. Acini filled with colloid, quite vacuolated, lining cells flattened cuboidal.

*Rat 554. Stock Diet.*—Gland near normal. Most acini contain smooth colloid and are lined by low or flattened cells. Some acini contain a granular material and the lining cells are large, cytoplasm granular, nucleus large and vesicular, cell outlines indistinct.

Marine (28) has described in detail the steps in the process of hyperplasia in the human thyroid. The histological findings in the goitrous glands of the rat are very similar to those reported by Marine for the human.

(c) *Comparison of fresh thyroid weights.*—The thyroid weights for the 193 rats receiving Diet GP yielded an average value of  $53.2 \pm .92$  mg. per 100 gm. body weight, with 24.1–126.0 mg. as the extremes of enlargement. Diet GPI yielded an average thyroid weight of  $12.6 \pm .19$  mg. per 100 gm. body weight with a range of 10.5–16.0 mg., while the stock ration<sup>6</sup> gave an average gland weight of  $12.9 \pm .25$  mg. per 100 gm. body weight with 9.0–18.6 mg. as the extremes. The average thyroid enlargement<sup>7</sup> was therefore 4.1–4.2 times the normal weight of the gland, while the extremes of enlargement were 2.3–7.9 times the normal weight. Based on these findings, it is apparent that Diet GP is markedly goitrogenic and is, therefore, a suitable diet for use in studies on goiter in the rat.

The largest gland removed actually weighed 117 mg. while, on the basis of 100 gm. body weight, the largest gland weighed 126.0 mg. When compared with the average normal thyroid weight, this latter gland weight would represent an enlargement of approximately 10 times.

The average thyroid weight of 20 stock rats at the beginning of the 35 day experimental period was  $16.4 \pm .32$  mg. per 100 gm. body weight, while

<sup>6</sup> Thirteen stock rats sent to us through the courtesy of Dr. C. N. Frey of the Fleischmann Laboratories, New York City, yielded an average moist thyroid weight of  $12.2 \pm .27$  mg. per 100 gm. body weight. The stock diet used was found by analysis in our laboratory to have an iodine content of 55 $\gamma$  per kilo.

<sup>7</sup> Enlarged thyroids were also found in 35 rats used in vitamin D assays and sent to us through the courtesy of Dr. Frey. These animals fed on the Steenbock rachitic diet yielded an average fresh thyroid weight of  $44.7 \pm 1.9$  mg. per 100 gm. body weight with 25.8 to 75.2 mg. as the extremes of enlargement.

at the end of the experimental period the value for rats on the same diet averaged  $12.9 \pm .25$  mg. This decrease in thyroid weight per 100 gm. body weight is accounted for by the fact that both in the rat (29-32) and in the human (28, 31) the thyroid is larger (on the basis of body weight) earlier in life.

When compared with the values given by Donaldson (33) for the thyroid weight of normal rats of the same body weight as our animals, our values

TABLE III  
THE INFLUENCE OF SEX ON THE WEIGHT OF THE THYROID GLAND IN NORMAL AND GOITROUS RATS

Diet	Iodine Content of diet $\gamma$ per kilo.	Number of rats			Fresh thyroid weight (in mg.) per 100 gm. body weight		Significance ratio
		Total	Males	Females	Males	Females	
Age = 59-66 days							
GP	15	193	102	91	52.0 $\pm$ 1.4 (24.1-114.0)	56.9 $\pm$ 1.9 (25.0-126.0)	2.0 Not significant
GPI	400	24	13	11	12.4 $\pm$ .20 (10.8-14.7)	12.8 $\pm$ .33 (10.5-16.0)	1.0 Not significant
Stock	47-72	39	21	18	12.9 $\pm$ .39 (9.0-18.6)	12.9 $\pm$ .30 (10.0-17.6)	0.0 Not significant
Age = 155-159 days							
Stock	47-72	20	10	10	9.6 $\pm$ .24 (8.5-14.5)	10.9 $\pm$ .23 (9.3-12.3)	3.9 Significant

for the weight of the gland obtained on the stock ration are lower. Thus, Donaldson's values for rats weighing 60 and 178 grams are 21.3 and 16.6 mg. per 100 gm. body weight, respectively, while our values at these body weights are 16.4 and 12.9 mg. On the other hand, McCarrison (34) obtained values for stock rats at these body weights which are lower than ours, i.e., 10.3 and 8.3 mg. per 100 gm. body weight. However, it is to be pointed out that our 60 and 178 gm. rats averaged only 27 and 62 days of age, respectively, whereas McCarrison's (35) animals at these body weights were 40 and 130 days of age. The smaller thyroid weights obtained by McCarrison may possibly be attributed therefore either to the poor growth of his rats, so that his animals in attaining these body weights had access

to the stock food for a longer period of time than our animals and hence were able to ingest more iodine, or to differences in the iodine content of the stock rations.

Figures 2 and 3 serve to show the differences in the size of thyroids obtained in our rats fed Diets GP and GPI.

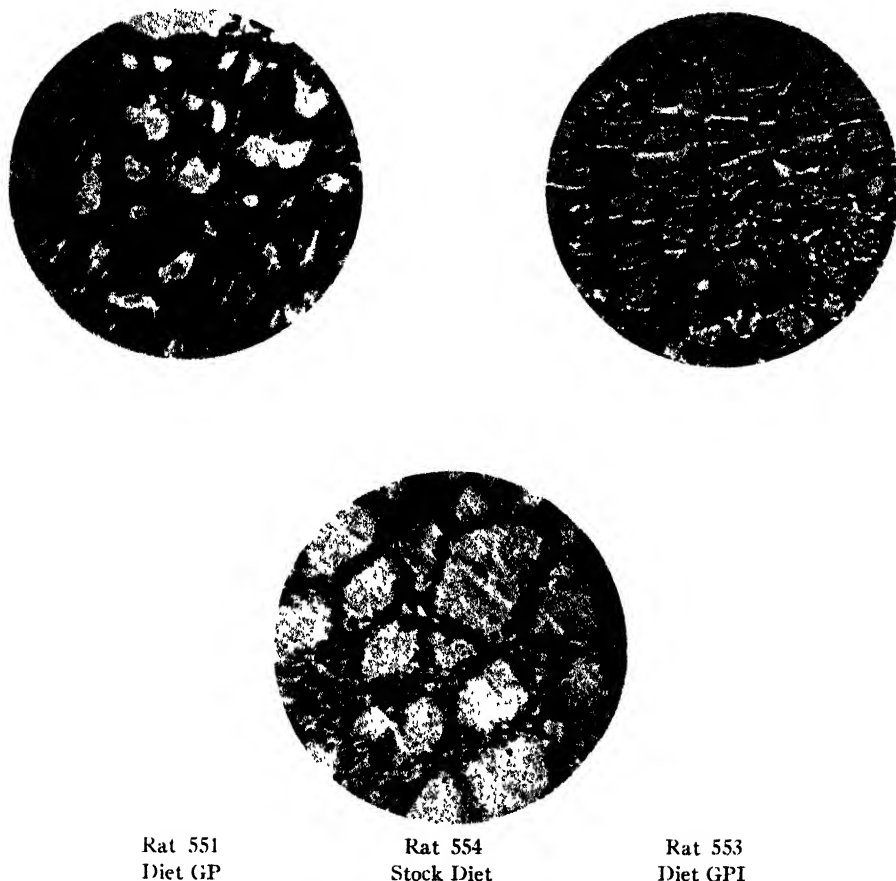


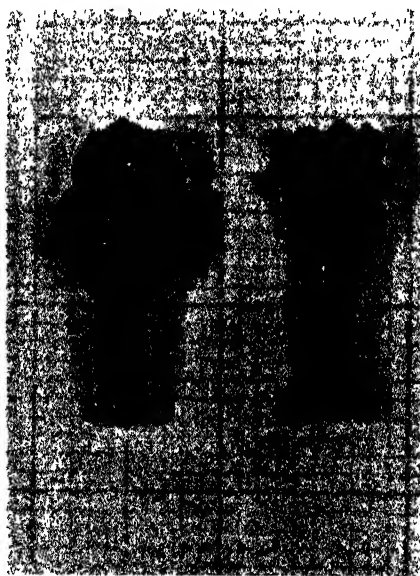
FIG. 1.—Showing the histological structure of the thyroid gland in three rats (litter mates) receiving various diets for 35 days. The goitrogenic diet (GP) contained  $15\gamma$  iodine per kilo. of diet; Diet GPI representing the goitrogenic ration containing added iodine, had an iodine content of  $400\gamma$  per kilo., while the stock ration contained  $47-72\gamma$  of iodine per kilo. Note the hyperplasia and lack of iodine-containing colloid in Rat 551 in contrast to the normal cell structure and presence of colloid in Rats 553 and 554.

(d) *Comparison of the dry thyroid weights.*—Table II also demonstrates that the thyroid enlargement obtained on Diet GP is primarily an actual gain in tissue weight and not merely an accumulation of fluid. Thus, the



dried goitrous glands weighed 9.9 mg. per 100 gm. body weight while the stock diet and Diet GPI yielded glands weighing 3.0 mg. and 3.5 mg. respectively. On a dry basis, the enlargement was therefore 3.3 and 2.8 times.

(e) *Comparison of the dry matter content of the glands.*—The goitrous glands contained only 19.0 per cent dry matter in contrast to 23.3 per cent for glands of rats on the stock ration and 28.0 per cent for glands of animals consuming Diet GPI, indicating that in the process of enlargement there is



Rat 551  
Diet GP

Rat 553  
Diet GPI

FIG. 2.—Showing the influence of iodine in the prevention of goiter. The photograph shows the glands attached to the excised tracheae. Rat 551 received the goitrogenic Diet GP while Rat 553 was fed Diet GPI for a period of 35 days. The actual fresh thyroid weights were 61.2 and 13.5 mg., respectively, while the fresh gland weights per 100 gm. body weight were 52.8 and 13.5 mg., respectively. The two rats were litter mates.

a definite accumulation of fluid. From this finding and the results just described relative to the extent of enlargement, it can be concluded that the production of goiter on Diet GP involves essentially a gain in tissue weight with the moisture content of the gland making a smaller but definite contribution. Oswald (36) also reported a higher water content in hyperplastic glands as compared with normal thyroids. By calculation from Marine's data (37) on the dog, the same finding is established.

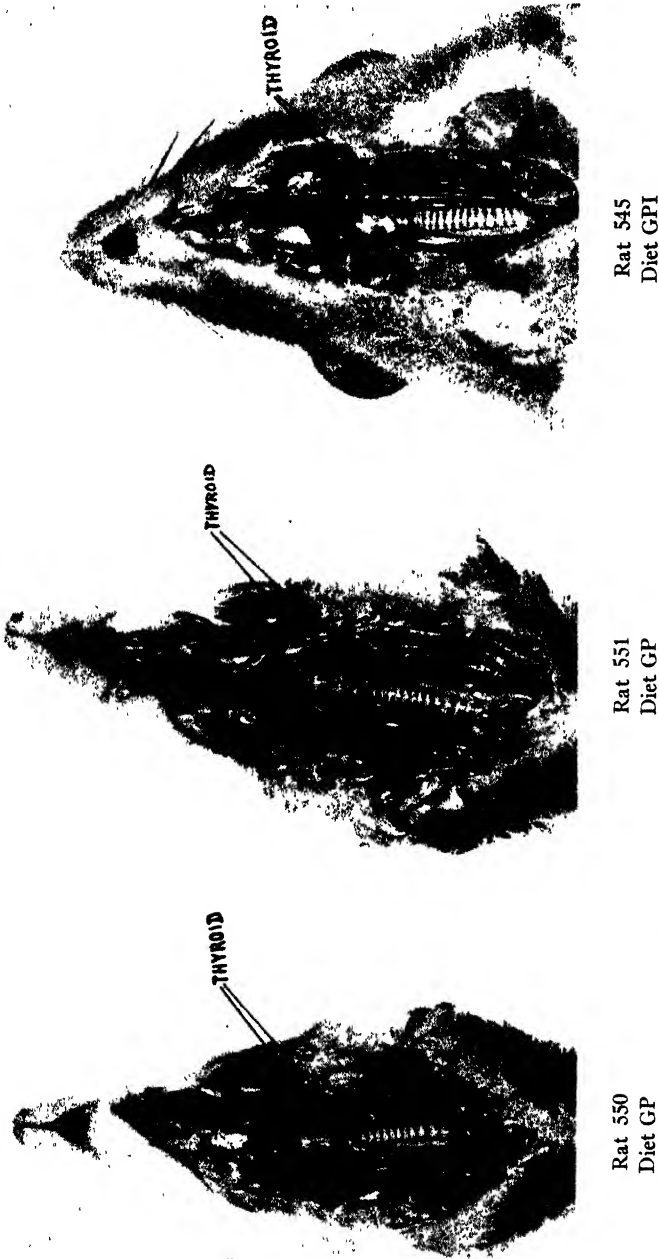


FIG. 3.—Showing the head and neck area with both normal and goitrous glands *in situ*. Rats 550 and 551 were fed the goitrogenic ration GP while Rat 545 was fed Diet GPI for a period of 35 days. Note the distention of the blood vessels leading to the thyroid in Rat 551. The actual fresh thyroid weights for Rats 550, 551 and 545 were 56.6, 61.2, and 11.7 mg., respectively, while the fresh gland weights per 100 gm. body weight were 57.8, 52.8 and 11.7 mg., respectively.

This finding that the goitrous glands were lower in dry matter content was confirmed later in our studies on the iodine requirement of the rat to be described in the following paper.<sup>8</sup>

(f) *Comparison of the iodine concentration in the glands.*—The goitrous glands were lowest in iodine concentration, i.e., 0.0083 per cent (dry basis) or 0.0016 per cent (fresh basis). These glands contained only approximately 1/8 as much iodine (dry basis) or about 1/9 as much (fresh basis) as glands from rats fed the stock diet for 35 days. When compared with glands from animals fed the iodine-containing Diet GPI, these values become approximately 1/32 and 1/47, respectively.

At the start of the experimental period, the glands contained an average of 0.0428 per cent iodine (dry basis) or 0.0110 per cent (fresh basis). In the production of goiter during the experimental period there was therefore a considerable decrease in the iodine content of the gland, the thyroids containing only 1/5 (dry basis) or 1/7 (fresh basis) as much iodine at the end as at the start of the experimental period.

In the human, Marine (28), who has correlated the histological appearance of the thyroid with its iodine content, reports an average iodine content of 0.0328 per cent dry basis (range, 0.0118–0.0584 per cent) for “marked glandular hyperplasia.” In the dog, Marine and Williams (37) found an average iodine content of 0.0114 per cent (dry basis) for glands with marked hyperplasia. These goitrous glands in the dog weighed approximately 4.2 times the normal, which represents practically the same extent of glandular enlargement that we are dealing with here in the rat. Our average value of 0.0083 per cent (dry basis) for the iodine content of the goitrous gland of the rat would indicate therefore that we are dealing with a well-advanced stage of goiter.

McCarrison (35) reports 0.0182 per cent (fresh basis) as the mean iodine content of the thyroid of his stock rats. This value is higher than our value of 0.0147 per cent (fresh basis) representing the average iodine content of the thyroid of our stock rats at the end of the experimental period.

(g) *Comparison of the total iodine content of each gland.*—A study of Table II reveals that the goitrous glands contained the least amount of iodine per gland, while Diet GPI yielded thyroids with the highest iodine content.

The actual average total amount of iodine in each gland at the start of the experimental period was 1.11  $\gamma$ . At the end of the experimental period, in rats on the goitrogenic ration this had fallen to 0.88  $\gamma$ , while on the stock diet it increased to 3.36  $\gamma$ . The goitrous rats ingested 0.14  $\gamma$  of iodine per

<sup>8</sup> *This Journal*, p. 347.

day or a total of  $4.9\gamma$  for the entire experimental period. It is evident therefore that this intake of iodine is insufficient to provide for the normal increase in iodine content of the thyroid gland coincident with advancing age and body weight (32, 35), with the result that thyroid enlargement or goiter develops as a compensatory process.

(h) *Comparison of the iodine intakes.*—The goiter-producing diet which was lowest in iodine content ( $15\gamma$  per kilo.) afforded the lowest intake of iodine, i.e.,  $0.14\gamma$  per rat per day. The stock ration which, as indicated in Table I, varied in iodine content from 47 to  $72\gamma$  per kilo. due to the purchase of new batches of ingredients from time to time, furnished an average daily iodine intake of  $0.74\gamma$ , approximately 5.5 times the amount provided by Diet GP. Diet GPI containing  $400\gamma$  of iodine per kilo. yielded an average daily intake of  $3.72\gamma$ , approximately 27 times the amount furnished by the goitrogenic ration.

(i) *Influence of Sex on Thyroid Weight.*—Since it is generally recognized that goiter in the human (28, 31) occurs with greater frequency in the female than in the male and that female glands are larger than male glands, we analyzed the data included in Tables I and II in order to determine whether there were any sex differences in the gland weight of normal and goitrous rats. Table III, which serves to show the results obtained, reveals the fact that among neither normal nor goitrous glands of young rats was significant sex difference in the weight of the glands apparent. In this connection, it is to be emphasized that these rats were only 59 to 66 days of age at autopsy and hence had not reached full sexual maturity. The influence of sex, therefore, would hardly have had an opportunity to assert itself. In order to determine whether older and sexually mature rats would show a sex difference in thyroid weight, three litters of rats containing 10 males and 10 females raised on the stock ration were killed at 155 to 159 days of age. The males averaged 313 gm. in body weight and the females 207 gm. The average thyroid weights are given in Table III. When the averages,  $9.6 \pm .24$  and  $10.9 \pm .23$  mg. per 100 gm. body weight were treated statistically, the difference of 1.3 mg. in gland weight was found to be significant. This finding indicates that in sexually mature normal females fed our stock ration the gland is slightly though significantly heavier than in male rats of the same age.

## DISCUSSION

In 1928, Marine (38) wrote:

“As a result of the numerous studies on the relation of iodine to the

thyroid gland our present views regarding the cause of goiter assume that it is a compensatory or work hypertrophy of the thyroid depending upon a relative or absolute deficiency of iodine. This deficiency of iodine may be due to: 1.—Factors which bring about an abnormally low intake of iodine. 2.—Factors which interfere with the absorption or utilization of an otherwise adequate intake. 3.—Factors which increase the needs of the body for the iodine-containing hormone." Accordingly, several possible explanations may be offered concerning the manner in which the goitrogenic Diet GP operates to produce thyroid enlargement and hyperplasia in the rat. This diet, as previously stated, is low in iodine content, yielding an average daily iodine intake of only 0.14  $\gamma$ . Goiter could develop on this diet in several possible ways: 1.—the amount of iodine furnished in the ration may be insufficient, when absorbed into the blood stream, to provide for normal thyroid function; 2.—the iodine may be present in the diet in a form unabsorbable or poorly absorbed in the intestinal tract; 3.—the iodine, if absorbed into the blood stream, may not be in a form utilizable by the thyroid gland to elaborate thyroxin; 4.—a combination of an inadequate iodine intake and poor absorption or utilization of this iodine, or 5.—the nature of the diet itself or some factor in the diet may bring about an increased demand for iodine. Webster and co-workers (39) and McCarrison (40) have shown that the ingestion of cabbage or certain other vegetables by rabbits creates an increased demand for iodine, resulting in goiter. In a review of the literature, Marine (41) points out that meat, liver and fat have been shown to be capable of producing thyroid hyperplasia in various animals, while Stott and co-workers (42) report that an excess of calcium in the drinking water is a causative agent in the production of goiter in human beings in India.

That the rat is able to utilize enough iodine from the goitrogenic ration to maintain a certain extent or degree of glandular enlargement is borne out in an experiment in which it was found that rats (litter mates) fed the diet for a period of either 35 or 124 days yielded essentially the same degree of thyroid enlargement. Further, in a preliminary basal metabolism experiment, it was found that four rats fed the goitrogenic diet for a period of eight weeks showed a basal metabolic rate of approximately 23 per cent below normal. When the animals were continued on the same ration for seven weeks longer, no further significant lowering of the metabolic rate occurred. These facts would seem to indicate that the goiter-producing ration GP is unable to furnish the rat with a supply of utilizable iodine sufficient to provide for normal thyroid function or the production of

adequate amounts of thyroid hormone, but does yield an amount of available iodine which limits the extent of thyroid enlargement and hyperplasia and then apparently maintains a subnormal functioning of the gland. For these reasons, we are making further efforts to devise a goitrogenic diet containing an even lower content of iodine so that even more severe goiter may be obtained.

In order to throw some light on the exact manner in which goiter develops in the rat, it would be necessary to follow carefully the paths of excretion of the iodine contained in the goitrogenic ration by carrying out metabolism studies. Such a procedure would simultaneously determine the availability of the small amount of iodine in this diet. By including, in such a study, rats fed on our stock ration and on Diet GPI representing a ration containing an adequate amount of iodine, further data concerning the metabolic behavior of iodine could be obtained. It is hoped to carry out such studies in a future investigation.

The Steenbock diet produces rickets in the rat by virtue of an abnormally high Ca:P ratio, i.e., approximately 4.3:1. This necessitates the inclusion of  $\text{CaCO}_3$  in the ration so that a high calcium content results. Stott and co-workers (42) believe that goiter in India is directly related to the high calcium content of the drinking water, while Hellwig (12) reports the production of goiter in the rat by means of  $\text{CaCl}_2$ . On the other hand, Simpson (43) found that the addition of lime to the diet of rabbits did not cause thyroid enlargement. For these reasons, we became interested in ascertaining whether the high calcium content or the abnormal Ca:P ratio of our goitrogenic diet may not be the cause of goiter in our rats or be one of the factors that bring it about. Preliminary results obtained on this phase of our investigations indicate that neither the high calcium content nor the abnormal Ca:P ratio are responsible for the goitrogenic activity of the diet. Further, by replacing the 3 per cent  $\text{CaCO}_3$  and 1 per cent NaCl in the goitrogenic ration with 4 per cent of Osborne-Mendel inorganic salt mixture (without KI) having a Ca:P ratio similar to that of cow's milk, we were still able to produce goiter. We are continuing our studies along this line, particularly because Baumann, Kurland, and Metzger (44) have recently reported that there is a definite retention of calcium, magnesium, and phosphorus in goiter produced in rabbits by cabbage feeding. When iodine is administered to cure such goitrous animals, there is a restoration of the balance of these elements by increasing the amounts excreted. As a result of this finding, Baumann and co-workers state that they "have reason for inferring that calcium retention is directly associated with the

development of simple goiter." Aub and his co-workers (45) report that the calcium excretion in myxedema is markedly diminished below that found in normal individuals.

The fact that the well-known Steenbock ration produces goiter both in the presence and in the absence of vitamin D has some bearing on the technic employed in the assay of foods for vitamin D. In such an assay, rats weighing 60 grams are placed on the Steenbock diet for a preliminary period of 18–21 days in order to produce rickets and are then fed for an additional ten-day period during which time the diet is supplemented with the food substance under test. Our results reported here show conclusively that in such a procedure, goiter as well as rickets is present at the end of the preliminary period, while in the curative period goiter may still be present while the rickets is being healed. If the vitamin D supplement happens to be cod liver oil, a rich source of iodine<sup>9</sup> then it is possible that both the rickets and the goiter may be cured at the same time. What effect the presence of goiter has on the prevention or cure of rickets we cannot say at this time. As yet we have no evidence to indicate that the presence of an adequate amount of iodine throughout such an assay would have any effect on calcification of bone and hence on the determination of the vitamin D potency of a food, but it is well to point out to investigators who are using the Steenbock diet in vitamin D assays, that the two dietary deficiency diseases are simultaneously present. Since in all types of biological assays the aim is to have only one dietary deficiency present, we therefore recommend that iodine be added to the drinking water of the rats throughout the vitamin test so that only rickets will be present. Although we hesitate to suggest at this time that iodine plays a rôle in bone metabolism, it is possible that the better clinical results (46, 47) obtained with cod liver oil as compared with viosterol both fed at the same number of vitamin D units may be due to the presence of iodine in cod liver oil and its absence in viosterol. Recently, Russell (48) in experiments on chickens has shown that the addition of vitamin A failed to increase the effectiveness of irradiated ergosterol sufficiently to make it equivalent in antirachitic potency to cod liver oil when the same number of vitamin D units of each substance was fed. This latter finding makes it appear that some factor other than vitamin A is responsible for the difference in antirachitic potency.

The question might be raised as to whether or not our stock ration—a

<sup>9</sup> Analyses in our laboratory show a very high iodine content for cod liver oil, i.e., approximately 10,000 $\gamma$  per kilo.

modification of the well-known Sherman breeding diet—contains an adequate amount of iodine. The thyroids from rats fed our stock ration did not have the pale pink color of the glands of rats fed Diet GPI, but were slightly red, despite the fact that the gland weights per 100 gm. body weights were practically the same on each diet; histologically, they were slightly below normal,<sup>10</sup> while the glandular iodine concentration—0.0634 per cent (dry basis)—was below the value of 0.1 per cent (dry basis) or 1 mg. iodine per gram of dried gland which Marine (41) states is the minimum amount necessary for the maintenance of normal gland structure. It is, however, possible that the ingestion of 0.74  $\gamma$  of iodine daily by our rats on the stock ration is adequate since good reproduction, lactation, and growth have been obtained in our breeding colony for many generations. That good growth also resulted in our experimental animals on this ration is evidenced by the fact that the rats attained an average body weight of 62 gm. at 27 days of age and an average body weight of 178 gm. at the age of 62 days. This rate of growth compares quite favorably with that reported by other workers using different stock diets.<sup>11</sup> Whether even better growth and reproduction would result if our stock diet were supplemented with iodine is not known. We hope to investigate this aspect of iodine metabolism soon.

The use of Diet GP in goiter studies would seem to possess several advantages over the cabbage feeding technic proposed by Webster (39). Diet GP produces in the rat an average thyroid enlargement of 4.1–4.2 times the normal gland weight in a period of 35 days. All five constituents of the diet are present as a composite mixture of dry ingredients which are inexpensive and are easily obtained in large enough quantities to last over an extended period of time. On the other hand, the cabbage must be steamed and then fed in a definite quantity each day. Again, it is definitely known that the goitrogenic activity of cabbage varies with the season of the year, and with the locality. This fact would make it necessary to preserve large quantities of the same lot of fresh cabbage for an experiment lasting for a long period of time. The fact that rats can be bred in large numbers on a standardized

<sup>10</sup> Mendel and Vickery (49) report that "rats raised in our laboratory on diets without any additions of iodine other than that present in the natural foods and drinking water used showed somewhat defective thyroids." Likewise, Thompson (21) reports abnormal thyroids in rats on a breeding ration.

<sup>11</sup> A sample of the stock diet used at the Fleischmann Laboratories was sent to us by Dr. Frey. It was found to contain 55 $\gamma$  per kilo. of iodine. This value compares quite well with the value of 47–72 $\gamma$  per kilo. for our stock ration.



stock ration insures a continuous supply of uniform animals and hence uniform results in experimental work.

### SUMMARY

A dietary technic suitable for goiter studies employing the rat as the experimental animal has been described. The goiter-producing diet used is low in iodine, having an average content of 15  $\gamma$  per kilo., and yielding an average daily intake of 0.14  $\gamma$  per rat.

A total of 193 rats were fed the goitrogenic ration for a period of 35 days. The following effects on the thyroid gland were observed: 1.—dark red color; 2.—marked hyperplasia with little or no iodine-containing colloid; 3.—average fresh weight of  $53.2 \pm .92$  mg. per 100 gm. body weight (range, 24.1–126.0 mg.), representing an average enlargement of 4.1–4.2 times the normal gland weight with 2.3–7.9 as the extremes of enlargement; 4.—a low dry matter content—19.0 per cent; 5.—a low concentration of iodine in the gland, i.e., 0.0083 per cent (dry basis) or 0.0016 per cent (fresh basis); and 6.—a low total iodine content per gland of 0.88  $\gamma$ .

The production of goiter on this ration involves essentially a gain in tissue weight of the gland with the moisture content of the gland making a smaller but definite contribution.

A total of 24 rats were fed the goitrogenic ration plus added KI to furnish approximately 3.72  $\gamma$  of iodine per rat daily, yielding the following results on the thyroid gland at the end of 35 days: 1.—normal pale pink color; 2.—normal histological structure with an abundance of colloid; 3.—an average fresh weight of  $12.6 \pm .19$  mg. per 100 gm. of body weight (range, 10.5–16.0 mg.); 4.—a dry matter content of 28.0 per cent; 5.—an iodine concentration of 0.2671 per cent (dry basis) or 0.0745 per cent (fresh basis) and 6.—a total iodine content per gland of 10.28  $\gamma$ .

Comparable results for the thyroid are also given for 39 rats fed on a stock ration containing 47–72  $\gamma$  of iodine per kilo.

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## STUDIES ON THE RELATION OF DIET TO GOITER\*

### II. THE IODINE REQUIREMENT OF THE RAT†

*By*

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AFTER we had developed a suitable dietary technic (1) for goiter studies using the rat as the experimental animal and had also gained some knowledge concerning the weight, iodine content, dry matter content, etc., of the normal and the goitrous rat thyroid, we next undertook to determine the iodine requirement of the rat. We are interested in this phase of our goiter research for two reasons; first, we feel that by determining the minimum iodine requirement of the rat we may be able to throw some light, indirectly, on the question of what constitutes the human requirement for iodine, a subject which lacks much needed direct experimental evidence; and second, we are anxious, in later studies, to compare the availability of various forms or sources of iodine, such as plant and animal iodine, di-iodotyrosine, thyroxin, iodized salt, potassium iodide, etc. In order to attack this latter problem adequately, it was considered necessary to feed the various sources of iodine in amounts *at or near* the minimum iodine requirement of the rat so that small differences in availability or utilizability, if present, may appear. This same principle of making comparisons at or near the minimum requirement is used in many studies in nutrition where comparisons of different sources of various dietary essentials are to be made, i.e., vitamins, inorganic elements, proteins, etc. In practically all earlier experimental feeding of iodine compounds to animals, the amounts used have been enormously greater than the physiological requirement (2-9).

Accordingly, we have made a study of the iodine requirement of the rat and have carried out two groups of experiments involving a total of 100 rats.

\* Aided by a grant from the Honorable Bernard M. Baruch.

† Presented before the American Society for Experimental Pathology, Philadelphia, Pa., April 27-30, 1932.

## EXPERIMENTAL

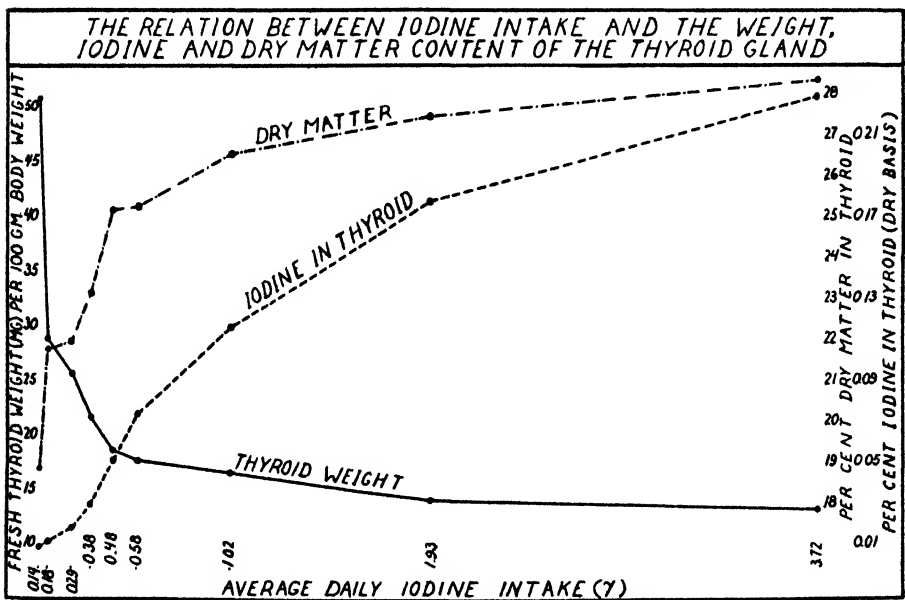
In the development of a dietary technic for the study of goiter in the rat (1), we found that the goiter-producing ration (Diet GP) furnished an average daily iodine intake of 0.14  $\gamma$ . It was evident, therefore, that a daily intake of iodine greater than this amount was necessary to prevent thyroid enlargement *on this diet*. Accordingly, in our first group of experiments we prepared a series of five diets containing different amounts of iodine, by evaporating various amounts of KI on Diet GP. These five rations—Diets GP, GPE, GPF, GPG and GPH—ranged in iodine content from 15  $\gamma$  per kilo. for Diet GP to 400  $\gamma$  per kilo. for Diet GPH (See Table I), yielding average daily iodine intakes per rat of 0.14, 0.59, 1.02, 1.93 and 3.72  $\gamma$ , respectively. These diets were fed to groups of ten rats each with the exception of Diet GP which was fed to a group of 20 rats. Ten litters of rats raised on our stock ration (1) having an iodine content of 47–72  $\gamma$  per kilo. were employed. The animals weighed approximately 60 gm. and were distributed so that litter mates were represented in each of the five groups of rats. The sexes were also divided evenly as far as possible. The experimental period lasted 35 days, at the end of which time the rats were killed and the thyroids removed. The fresh thyroid weight was immediately determined after which all the glands of rats on the same diet were pooled and dried to constant weight at a temperature of 80°C. The average iodine concentration in the glands was determined by a modification of the micro-McClendon method (10).

Based on our findings in this first group of experiments, it was evident that a study of the influence of iodine intakes lower than 0.59  $\gamma$  daily would yield important information. Accordingly, we carried out a second group of experiments in which four additional diets—Diets GPA, GPB, GPC and GPD—were fed. These rations ranged in iodine content from 20–51  $\gamma$  per kilo., yielding intakes of 0.18, 0.29, 0.38, and 0.48  $\gamma$  of iodine per day, respectively (see Table I). In this experiment, 40 rats, evenly divided as to litter and sex, were used.

The results of these two groups of experiments are given in Table I and in Chart 1. From a study of these data, it is at once evident that as the iodine intake increased, there was a corresponding decrease in the thyroid weight, while the iodine concentration in the gland, the total iodine content of each gland, and the dry matter content of the gland increased. In other words, as goiter develops the moisture content of the thyroid increases, while the iodine concentration in the gland decreases. Apparently the so-called "thyroid colloid" which fills the acini of normal glands has a

lower water-holding power than the cytoplasm of basement cells, which in goitrous glands are found to be much enlarged. In our previous study (1), we reported that goitrous glands were dark red, in contrast to normal thyroids which were pale pink in color. In the present experiment, we observed that as the daily iodine intake increased with the feeding of the various rations, there was a simultaneous lessening in the intensity of the red color of the gland until the normal pale pink color was reached. The increased

Chart 1.



blood supply of goitrous glands may be a factor in the observed increase in water content.

From the results of these two groups of experiments, the following facts are revealed:

An inverse relationship exists between; 1.—the iodine intake and the weight of the thyroid gland; 2.—the iodine concentration in the gland and the weight of the thyroid; 3.—the dry matter content and the weight of the thyroid; 4.—the iodine intake and the intensity of the color of the gland.

A direct relationship exists between; 1.—the iodine intake and the iodine content of the thyroid; 2.—the iodine intake and the dry matter content

**TABLE I**  
**A SUMMARY OF DATA ON THE IODINE REQUIREMENT OF THE RAT, SHOWING THE RELATION BETWEEN IODINE INTAKE AND THE WEIGHT, IODINE AND DRY MATTER CONTENT OF THE THYROID GLAND**  
 (All rats started at approximately 60 g. body weight\* and fed experimental diets for 35 days)

Diet	Iodine content of diet γ/kilo.	Iodine intake		No. of rats	Initial and final body wts.	Dry matter in thyroid	Average thyroid weight						Iodine conc. in gland		Total iodine in each gland	
		Av. daily	Av. total				Fresh			Dry			Dry basis	Fresh basis	Actual	per 100 gm. body wt.
							Actual	per 100 gm. body wt.	mg.	Actual	per 100 gm. body wt.	mg.				
		γ†	γ		gm.	%	mg.	mg.	mg.	%	%	γ	γ	Actual	per 100 gm. body wt.	
GP	15	0.14	4.9	20	62-114	18.8	57.7 (32.7-97.7)	50.6±2.6 (27.7-84.4)	10.8	9.5	.0070	.0013	0.76	0.67		
GPA	20	0.18	6.4	10	63-115	21.7	32.9 (20.8-44.9)	28.6±1.3 (21.5-41.3)	7.1	6.2	.0102	.0022	0.73	0.63		
GPB	31	0.29	10.3	10	60-114	21.9	29.1 (24.7-34.9)	25.5±.80 (19.9-31.2)	6.4	5.6	.0165	.0036	1.05	0.92		
GPC	41	0.38	13.3	10	60-111	23.1	23.8 (16.8-33.6)	21.4±.84 (16.2-26.0)	5.5	4.9	.0282	.0065	1.55	1.39		
GPD	51	0.48	16.8	10	61-114	25.1	20.9 (15.3-26.8)	18.4±.61 (14.9-24.4)	5.3	4.6	.0494	.0124	2.53	2.28		
GPE	63	0.59	20.7	10	61-115	25.2	19.9 (17.0-24.5)	17.5±.41 (14.8-21.5)	5.0	4.4	.0729	.0184	3.57	3.21		
GPF	110	1.02	35.7	10	63-106	26.5	17.3 (14.0-20.9)	16.4±.49 (13.5-20.5)	4.6	4.3	.1150	.0305	5.27	4.94		
GPG	207	1.93	67.6	10	63-110	27.4	15.4 (11.7-19.5)	14.0±.44 (11.9-18.8)	4.2	3.8	.1765	.0484	7.43	6.71		
GPH	400	3.72	130.2	10	64-114	28.3	14.9 (11.4-17.7)	13.1±.36 (10.5-16.0)	4.2	3.7	.2275	.0644	9.60	8.42		

\* Average age = 27 days.

of the gland; 3.—the iodine concentration in the gland and the dry matter content.

A further study of the chart reveals the fact that the area represented by iodine intakes ranging from 0.14–0.59 $\gamma$  is a very sensitive or critical one, in that small increments or decrements in iodine intake in this area result in marked abrupt changes in the weight, iodine concentration, and dry matter content of the thyroid. On the other hand, for iodine intakes between 0.59–3.72  $\gamma$ , the effect on the gland is much less marked, particularly for the higher intakes in this range, indicating thereby a tendency of the thyroid to reach constant values.

The highest iodine intake—3.72  $\gamma$ —yielded thyroids with an iodine concentration of 0.2275 per cent (dry basis) and an iodine content per gland of 9.60  $\gamma$ . It would be interesting to determine the maximum amount of iodine the rat thyroid is capable of storing.

The point at which the actual physiological need for iodine is fulfilled and storage of iodine begins can be approximated from our data. From the study of a large number of thyroids from different animals, Marine (11) has reported that when the iodine concentration in the thyroid was below 0.1 per cent (dry basis), hyperplastic changes were regularly found in the gland. If we consider, therefore, 0.1 per cent as the minimum effective level of iodine, then by interpolation in Chart I, approximately 0.90  $\gamma$  iodine daily would appear to be the minimum iodine requirement of the rat when fed on the goiter-producing ration GP. This intake of iodine corresponds to a fresh thyroid weight of about 16.5 mg. per 100 gm. body weight and a dry matter content of approximately 26.0 per cent. However, it is evident from a study of our data, that daily iodine intakes greater than 0.90  $\gamma$  yielded glands lighter in weight than 16.5 mg. per 100 gm. body weight and containing a higher concentration of iodine and a higher dry matter content. It would seem, therefore, that a better criterion for the minimum iodine requirement would be the *smallest amount of iodine necessary to prevent any significant glandular enlargement*. In our experiments, Diets GPF and GPG furnishing 1.02 and 1.93  $\gamma$  of iodine daily yielded thyroid weights of  $14.0 \pm .44$  and  $13.1 \pm .36$  mg. per 100 gm. body weight, respectively. When statistically treated, the difference of 0.9 mg. in thyroid weights in these two groups was found to be not significant. Using the above criterion, therefore, the minimum daily iodine requirement of the rat when fed the goitrogenic ration would be approximately 1–2  $\gamma$  iodine, this intake of iodine corresponding to a glandular iodine concentration of approximately 0.11 to 0.18 per cent (dry basis).



Obviously, more accurate data, particularly histological findings, are needed in order to locate more definitely the minimum effective level of iodine. We are continuing this phase of our research and are planning to correlate thyroid weight, iodine and dry matter content with histological findings at various intakes of iodine.

### DISCUSSION

Views as to human iodine requirement have up to this time been based almost entirely on analysis of diets in goitrous and non-goitrous regions, or on the iodine elimination of individuals in such regions. Summarizing such available information, Cameron (12) states that the minimum quantity of iodine required has been estimated to amount only to between 35 and 70  $\gamma$  per day, while Orr and Leitch (9) state that the minimum daily requirement may be about 45  $\gamma$  for an adult male and 150  $\gamma$  for a child. If it is assumed that the average daily caloric intake is 50 calories for the rat and 3,000 calories for the average human, then a calculation based on the relative caloric intake gives a factor of 60. Taking the iodine requirement of the rat at 1–2  $\gamma$  per day, the human requirement would then amount to approximately 60–120  $\gamma$  per day, or if we take the value of 0.9  $\gamma$  daily for the rat as the minimum amount of iodine necessary to provide 0.1 per cent of iodine (dry basis) in the rat thyroid, this value would yield a corresponding value of approximately 54  $\gamma$  for the human. Such a method of comparison will doubtless be criticized, but is certainly more reliable than calculations based on relative body weight, as is sometimes done in toxicity studies. When the results of these calculations are compared with the estimates given above by Cameron and Orr and Leitch, the agreement is striking.

That very little is known regarding the iodine requirement of animals is evident from a recent review of the literature by Orr and Leitch (9). Thus, these investigators state: "Regarding the iodine requirements of animals we have no definite information. Nor can we deduce the requirement of farm animals from a knowledge of the amount required to prevent goiter, for as yet no standard and adequate dosage has been determined. The doses recommended by different workers vary widely and are probably all unnecessarily high." Until precise data are obtained for different species of animals *we suggest, therefore, that 20–40  $\gamma$  per 1,000 calories of the ration be considered as the minimum iodine requirement for farm animals.*

It is a common practice in most laboratories employing synthetic diets in experiments on rats to include 4 per cent of Osborne-Mendel inorganic salt mixture (13) in order to furnish all the essential mineral elements, in-

cluding iodine. This salt mixture contains 0.0025 per cent of iodine so that a daily intake of 10 grams of food yields 10  $\gamma$  of iodine. Mendel and Vickery (14) raised the question as to whether this amount of iodine is sufficient for normal growth in the rat. They investigated the possible desirability of increasing the iodine content of this salt mixture and found no improvement in growth of rats when additional iodine (equivalent to 844  $\gamma$  daily as KI) was added to a synthetic diet already containing 4 per cent of the salt mixture. Considering our own findings on the iodine requirement of the rat, it is evident that 10  $\gamma$  of iodine daily provides a quantity of this element from five to ten times the minimum need and is therefore undoubtedly adequate for normal functioning of the thyroid gland.

The fact that the rat requires such a small amount of iodine may explain the inability of some investigators to produce goiter in the rat by diet. Thus, Jackson and P'an (15) who failed to produce goiter on a low-iodine synthetic ration report<sup>1</sup> that the iodine content of their ration approximated 1  $\gamma$  per day. Likewise, Hellwig (16) who concluded from his experiments on rats that insufficiency of iodine is not the essential cause of goiter may have fed his animals enough iodine to prevent goiter, for he assumed, without analysis, that the iodine content of his experimental rations was low.

#### SUMMARY

The minimum iodine requirement of the rat was determined by feeding various groups of rats on a goitrogenic diet to which different amounts of iodine (as KI) were added. Using as a criterion *the smallest amount of iodine necessary to prevent any significant thyroid enlargement*, the minimum iodine requirement was found to be approximately 1–2  $\gamma$  per rat per day. This intake of iodine yielded a concentration of 0.11–0.18 per cent iodine (dry basis) in the thyroid.

The daily intake of iodine necessary to yield a concentration of 0.1 per cent iodine (dry basis) in the thyroid was found to be approximately 0.9  $\gamma$ .

The inclusion of 4 per cent Osborne-Mendel salt mixture in synthetic diets for experiments with rats provides from 5–10 times the minimum iodine requirement of the rat.

Using for comparison the results obtained in the rat, the minimum iodine requirement of the human was calculated to be approximately 60–120  $\gamma$  per day.

<sup>1</sup> Personal communication.

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## THE EFFECTS OF IRRADIATED ERGOSTEROL ON THE METABOLISM OF NORMAL DOGS\*

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IN THE course of experiments designed to elucidate the problem of the mechanism of action of irradiated ergosterol (Reed and Thacker, 10) it was noted that if normal animals were given large doses over some time, they invariably lost weight. This suggested the possibility of involvement of protein metabolism and, therefore, the further possibility of an influence on basal metabolism. These possibilities seemed the more reasonable in view of the known low metabolic rate of rachitic subjects (Seel, 11).

Consequently, the major portion of the investigation herein to be reported has to do with the effects of irradiated ergosterol on basal metabolism and the correlation of these effects with alterations in nitrogen metabolism, and with the mobilization and excretion of calcium and phosphorus.

The dogs were kept in metabolism cages during the period of observation. A standard diet of beef heart and puppy meal, ratio of 3:1 by weight, was used throughout, each new portion being analyzed for nitrogen, calcium, and phosphorus. The weight of the food consumed by each dog was recorded carefully. Any residue after feeding was preserved and weighed. The detailed data for food consumption were not included in the graphs because this would only complicate the presentation and would not add materially to the discussion of food consumption included in the text. The food nitrogen was determined by the Kjeldahl method as described by Hawk and Bergeim (5), using 2 grams of dried food. For the inorganic constituents 10 grams of the dried food were placed in a vitrosil crucible and heated at 800°C. until only a white ash remained. This was then dissolved in nitric acid and evaporated to dryness. Next, the material was dissolved in 6 N hydrochloric acid, filtered and diluted to 100 cc. From this

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filtrate, calcium and phosphorus were determined by the methods described for urine.

Urine was collected daily at 9:30 a.m. and analyzed for total nitrogen by the Kjeldahl method, urea N by the Folin and Youngburg method, creatinine by the Folin method (3), calcium by the Shohl and Pedley method (12), and inorganic phosphorus by the method of Fiske and Subarow (2). Toluol was placed in the urine receptacles as a preservative.

The metabolism cages were cleaned three times each week and the feces saved. The feces collection from each cage for each week was dried and thoroughly mixed and three samples taken, ranging from 1.0 to 1.5 grams. Each sample was transferred to a Kjeldahl flask with 25 cc. of concentrated sulphuric acid, 25 cc. of water, and 5 grams potassium sulfate, and allowed to stand over night. The further procedure was the same as for urine. Ten gram samples of feces were weighed into large vitrosil crucibles and ashed at 800°C., after which the rest of the procedure was as described above for calcium and phosphorus.

The animals were bled before feeding or injecting. About 10–12 cc. of blood were drawn directly from the heart by needle and syringe, using heparin as an anticlotting agent. Plasma calcium was determined by Clark and Collip's modification of the Kramer-Tinsdall method. Inorganic phosphorus was determined by the Fiske and Subarow method (2). Kerr's procedure (7) for potassium was used.

At the termination of each experiment an autopsy was performed and tissues saved for chemical and microscopic studies. The tissues were weighed immediately, then dried at 105°C. for three days and again weighed into a vitrosil crucible and ashed at 800°C. The ash was dissolved in nitric acid and evaporated to dryness. The material was then dissolved in 10 cc. of 20 per cent trichloroacetic acid, and diluted to 25 cc. in a volumetric flask. Portions of this filtrate were used for calcium determination. The phosphorus was determined as in blood.

The basal metabolic rate was determined on trained dogs by the Sanborn-Benedict machine, as described by Kunde and Steinhaus (9). All the necessary precautions as to training and technic were observed, and food consumption standardized during the period of preliminary observation.

Calculation of surface area was made by the Meeh formula. While this formula is satisfactory for comparative purposes with subjects of constant weight, it is apparent that the loss of weight introduces a complication for which no satisfactory solution has yet been devised. In some of the figures are presented two graphs of metabolic rate, one based on the

daily weight fluctuations, the other on the original average weight over a period of days. In every instance the second graph shows a lower metabolic rate. The two graphs do not materially diverge, however, until after there has been considerable loss of weight. Consequently, the conclusions as to the early effects on the metabolic rate are not invalidated. In later stages the true metabolic rate will obviously lie somewhere between the two calculations.

Another possible source of error in these calculations lies in the fact that the figure 4.83 was used throughout as the calorific value of oxygen. This assumes a respiratory quotient of approximately 0.83. We believe that the quotient does not remain constant. This belief is grounded on the fact that the fat stores are completely exhausted after a time. In fact it seems probable now that most, if not all, of the early weight loss is to be ascribed to consumption of body fat. In that case the calorific value of oxygen would be expressed by a much lower figure and consequently the entire curve of metabolic rate would be appreciably lower than represented, except possibly in the earlier stages, before weight loss is manifest.

It has been shown that when ergosterol is administered by mouth there is likely to be some reduction to coprosterol in the intestinal tract, and therefore some loss in potency. Since the proportionate loss necessarily varies in different subjects and under variable conditions, it was deemed necessary first to adopt a method of administration that would insure constant potency of the material applied to the effector mechanism, whatever it may be. Furthermore Klein and Russel (8) have shown that it is possible to recover from the feces 26 to 43 per cent of irradiated ergosterol fed by mouth. Reed and Thacker (10) have demonstrated that the material in oil can be quantitatively administered by intravenous injections. This method was first applied in this laboratory in February, 1929. The question has been raised, frequently, as to the danger of oil emboli. In more than 1,000 individual injections, in this laboratory, of ergosterol in oil or of corn oil alone, in amounts up to 15 cc., to both dogs and human subjects, there have been no indications of emboli. Therefore it is believed that this is a safe and reliable method. Taylor (13) and his co-workers also report successful intravenous administration to animals.

To our knowledge, there have appeared in the literature only three reports of experiments on the effect of irradiated ergosterol on the metabolic rate. Seel (11) reported that this substance restored to normal the lowered metabolic rate of rachitic rats. Thöenes (14) found that there was an increased gaseous exchange in normal subjects that bore no quantita-

tive relation to dosage and that nitrogen exchange was increased. Handowsky (4) believed that ergosterol is a metabolic stimulant similar to saponin.

In a series of experiments on nitrogen balance in one human subject and five dogs, two of which were studied during two relatively long periods, it was impossible to demonstrate any constant variation in nitrogen metabolism. The total nitrogen excretion and balance, and the urea and creatinine excretion were subject only to such variations as might occur normally.

#### BASAL METABOLISM

Each dog was subjected to a long period of training and the average for the period after a fairly stable value had been established was taken as the basal rate. This training period was much longer than is shown in the charts, since the starting point on each illustrative case is taken as the day on which the animal was placed under strictly standardized conditions. For this reason in the foreperiod, as shown in the illustrations, the average of the rate is sometimes below the basal figure. This does not, however, invalidate the comparative results.

In Figure 1 (Dog No. 17) the basal rate was 800 cal./sq.m./24 hrs. The viosterol was actually administered as a 10,000 $\times$  preparation but the dosage was calculated as cc./kg.m. of 100 X. Total, 1498 cc., or 93.6 cc./kg. of original weight. It will be noted that there was at first a steady, consistent rise in the rate over five days, with considerable variation thereafter, but with an average rate well above basal. The sudden sharp fall that occurred after the 30th day was at that time interpreted as an indication of established tolerance, consequently the dose was doubled on the 34th day, but a new preparation was used. Subsequently it was found that the first preparation had deteriorated in potency.

With the higher dosage the rate promptly increased again, finally, on the 42nd day, reaching the high point of plus 85 per cent. With discontinuance of administration after the 43rd day the rate gradually declined to a normal level on the 52nd day. With a still larger dose of 3 cc., begun on the 54th day, the rise in rate was much less pronounced. By this time the animal was obviously in a toxic state so that the subsequent results are of doubtful value. Death in coma occurred on the 89th day.

As mentioned above, the calculations of surface area on the basis of weight alone is of questionable validity. In this chart the calculation on the basis of weight at the time administration was begun (16.3 kg.) gave a surface area of 0.72 sq.m. and the higher graph is based on this figure. The

cross-hatched area indicates the rate calculated on daily weight. At the termination of the experiment the dog weighed 11 kilos, from which the formula gives a surface area of 0.554 sq.m. This means a loss of 32.5 per cent in weight and 23 per cent in area. It is scarcely conceivable that the figures for variation of surface area can be correct. Hence the above statement that the true rate probably lies somewhere between the two. Except where indicated the calculations are based on the actual weight of the animals on that day. This discrepancy does not, however, invalidate the results in the early stages before weight loss became pronounced. In fact, in the first period, the high rate, while accompanied by some irregularity was not accompanied by any consistent weight loss. Up to the 48th day this animal showed considerable increase in appetite so that the weight loss was not due to lessened food consumption.

In all animals, at autopsy, it was found that all visible stores of body fat were exhausted. This fact suggests, therefore, that the weight loss is due in the early stages to increased consumption of body fat. As will be seen in other cases, the time of beginning weight loss varied. When delayed there was invariably an increase in food consumption. It seems possible, therefore, that the delay depended on the extent to which food consumption counterbalanced fat loss.

The blood studies in this case do not confirm the common statement that the inorganic phosphorus is increased by ergosterol. While there are high values at certain points they are not consistently so. This is true of all of the animals in this series. The values for potassium are likewise widely variable so that it cannot be concluded at present that the blood concentration of this substance is affected in a constant manner.

The blood calcium did not show any pronounced change until the 31st day and had not returned to a normal level in more than three weeks after the last dose.

In case of Number 24 (Fig. 2) 9 large doses—4.8 cc./kgm.—were injected over a period of 11 days. Total 518.4 cc., or approximately  $\frac{7}{4}$  43.2 cc./kgm. of original weight. The metabolic rate was immediately increased and sustained through 5 days after the last injection, after which it fell abruptly to basal. Subsequent fluctuations cannot be evaluated. No pronounced weight loss occurred until the second day of the afterperiod. Appetite was good until the 32nd day, after which there was little food consumed.

In Number 25 (Fig. 3) the initial dose was only 3.7 cc./kgm. daily, but the response was immediate and sustained. After 8 days the dose was re-



duced to 0.7 cc./kgm. While there was some fluctuation in the rate, the average level continued high, approximately plus 33 per cent. With further reduction to 0.35 cc./kgm. the rate was still sustained at a definitely high level to the end of the experiment. Total viosterol 544.6 cc. or 38.4 cc./kgm. original weight.

There was no increase in inorganic phosphorus and no immediate increase in blood calcium. Weight loss was not as great as in either of the preceding animals, the final loss being slightly less than 13 per cent.

Number 26 (no chart) was given 2.3 cc./kgm. daily over a period of 12 days, and 0.6 cc./kgm. over 5 days. Total 260 cc. or 30.6 cc./kgm. The metabolic rate increased immediately and remained about +40 per cent. There was no weight loss until the tenth day of administration. The final weight was 7.2 kilos. as against 8.5 kilos. initial weight, approximately 15 per cent loss. The results in this case and in Number 24 are comparable in every respect except that the experiment was terminated at an earlier date in the case of Number 26.

Number 29 (no chart) received 7.25 cc./kgm. daily over a period of 13 days, but in spite of the high dose the average level of metabolic rate was only +20 per cent. The dose was now reduced to 1 cc./kgm. daily over 13 days with the result that the rate increased promptly to +30 per cent and two days later to +50 per cent. The average during this period was approximately +35 per cent. This response to a lower dosage has been noted in several cases and is one of the puzzling features of this work. At present a possible explanation seems to be that the animals had become sensitized. An alternative explanation is that the heavy administration resulted in storage of ergosterol in the body so that some of it was liberated and acted along with the smaller dose subsequently administered.

The fact that the early metabolic level was not as high on high dosage as in the other cases on lower dosage confirms Thöenes' claim that there is no quantitative relation between dose and metabolic rate. Still further confirmation is found in others of our experiments.

Blood calcium rose from 10.20 mgm. to 29.10 on the 21st day and was still 29.20 on the 29th day. Phosphorus fell from 6.01 mgm. to 3.24 although on the 21st day the figure was 8.52. Potassium was practically constant throughout.

It is obvious that the rise in metabolic rate cannot be correlated directly with any other factor studied except in the most general way.

That the response is not always prompt is illustrated by Number 30 (Fig. 4). On the 9th day of observation this dog received a daily dose of

2.5 cc./kgm. but it was not until the 15th day that the metabolic rate increased 30 per cent. On the 16th day the dose was reduced to 0.8 cc./kgm. but a rate of +38 per cent was maintained through three days. Because of a technical difficulty no further metabolic rate determinations were made on this dog. Beginning with the 20th day the dose was further reduced to 0.45 cc./kgm. daily. All observations were discontinued after the 30th day (September 15, 1930).

No significant weight loss occurred until the 18th day. The final weight was 9 kilos. as compared to 12.3 at the beginning. This weight was maintained for about a month when there began a slow recovery until on December 13, 1930, the weight was 15.4 kgm. During this period of recovery, the metabolic rate was very high, gradually falling from +50 per cent to basal by December 15 (91st day). Again because of a technical difficulty it was necessary to discontinue metabolic rate determinations.

On January 8, 1931, was begun a balance experiment on this dog, part of the results of which are shown in Figure 5. Urine volume was increased in the after period. Urine phosphorus decreased slightly but only as phosphorus consumption diminished, a good balance being maintained throughout.

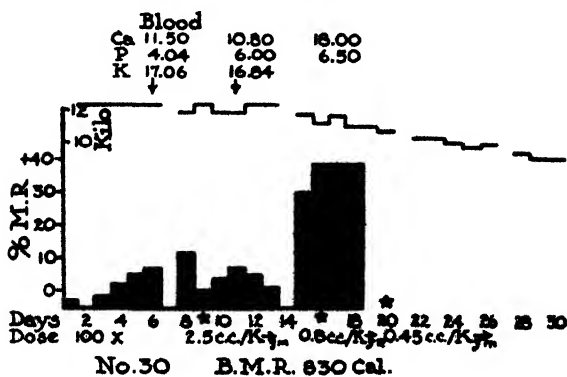
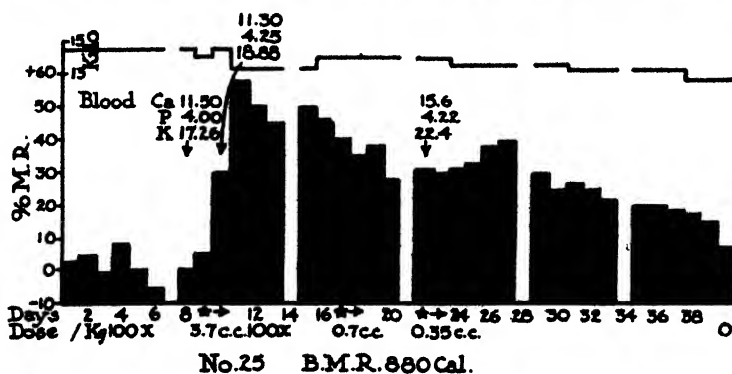
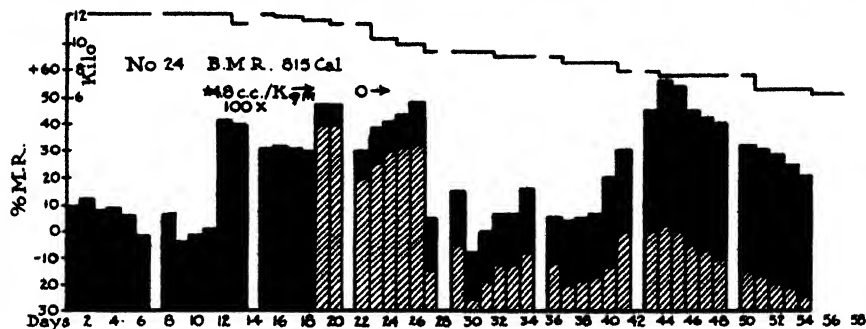
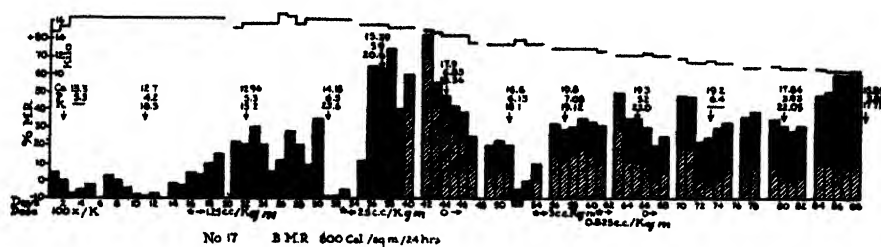
Urine Ca elimination was at a low level when administration was begun on the 32nd day but on the 37th day a progressively increased output became apparent, which continued 5 days after the last dose.

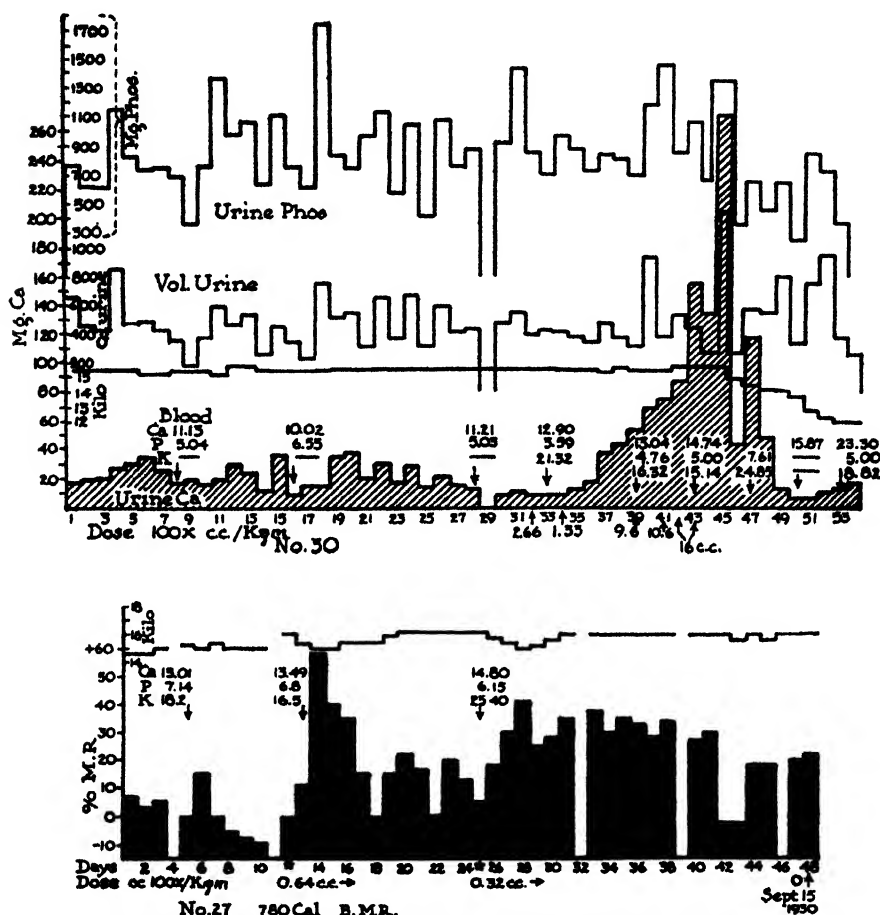
Blood Ca was increased progressively from 11.20 mgm. to 24.5 on the 4th day after the last dose, falling to 15.87 three days later but, strangely enough, rising again in three days to 23.30 mgm.

Weight loss was quite rapid after the 45th day, on which the highest urine Ca was found, the final figure being 12 kilos. nine days later. To what extent weight loss and high urine Ca output may be directly correlated remains to be determined.

In case of Number 35 (no chart) an effort was made to select a minimal effective dosage. On the 21st day 0.5 cc./kgm. daily was given but the metabolic rate showed no increase until the 25th day. There was no significant sustained weight loss. Urine P was slightly decreased during the period of administration. Urine Ca was not greatly affected, nor was the Ca balance altered. Total Ca elimination was decreased during the period of injection and slightly increased in the after period over the average of the foreperiod. It seems probable therefore that, for this dog, the dosage was about the threshold of toxicity.

Another experiment of the same kind was done on Number 27 (Fig. 6).





In the above Figures 1 to 6 inclusive, ★ indicates the date on which a given dosage was begun, e.g. Fig. 1, 16th day. The number of cc. per kilogram is shown by the figure accompanying. The arrow following indicates that that dose was repeated each day until another legend appears. A zero followed by an arrow indicates that on that date *et seq.*, no viosterol was administered.

Beginning with the 12th day 0.64 cc./kgm. daily promptly increased the metabolic rate 57 per cent. There were, however, three periods of subsidence to basal on the 18th, 22nd, and 25th days, which suggests that the dose was not sufficient to maintain a high metabolic level. This conclusion, however, is called into question when it is seen that half of this dose did sustain a high level, except for one remission through the 48th day.

Transient weight loss occurred at two points, both coinciding with peaks of the metabolic rate.

On the 106th day, November 13, 1930, the experiment was resumed (Figures 7 and 8). The metabolic rate had fallen to basal as had also the concentration of blood constituents. With the daily administration of 0.5 cc./kgm. begun on December 3, there was only slight stimulation of metabolism.

The average during the foreperiod, exclusive of the first two determinations, was 778 cal. per sq.m. per 24 hrs., approximating closely that for the foreperiod five months earlier. The average during this period of administration was 870 cal. approximately +15 per cent. This would not be a significant figure for any one day, but an average of this magnitude sustained over a week is believed to represent a definite effect. Even the three day period begun on December 20, over which 0.3 cc. daily were injected, seems to have stimulated the rate to some extent.

From December 22 to January 5, 1931, no viosterol was given. On the latter date the rate was 800 cal. With the administration of 0.6 cc. on this date *et seq.* there was a prompt rise which was well sustained. With reduction of the dose to 0.3 cc. there was some decrease in the rate. Following a two day interval with no viosterol the rate declined to 811 cal. but rose again with resumption of this dose for 3 days and fell off after 5 days to 751.

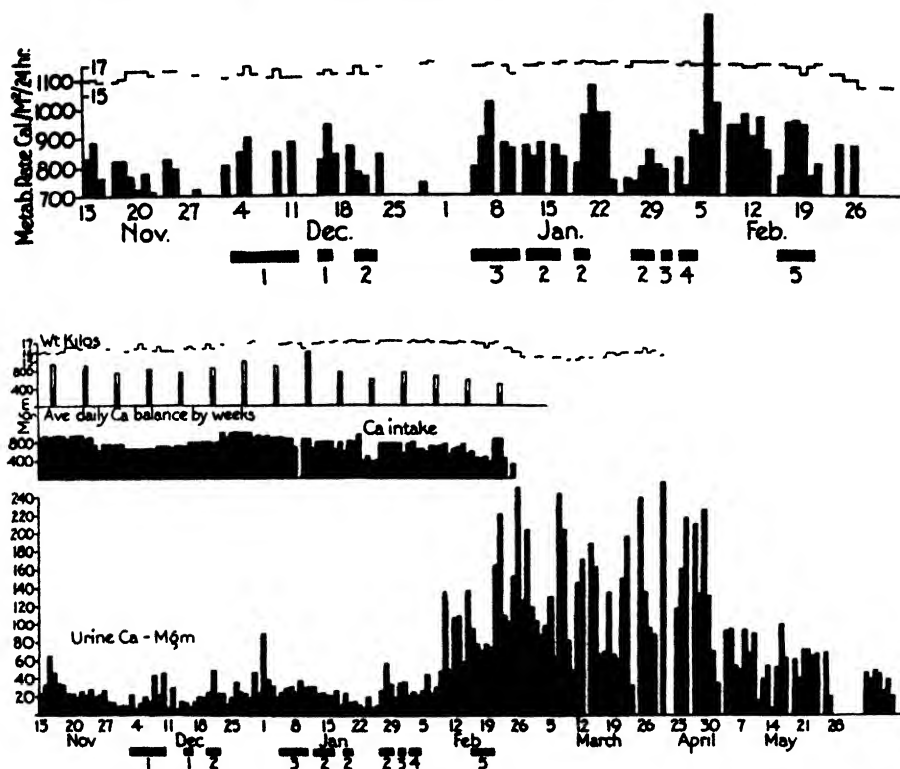
It is apparent that as small a dose as 0.3 cc. daily of 100 X viosterol per kilogram of body weight was sufficient to stimulate the metabolic rate of this animal, but that only slightly greater stimulation occurred on a dose of 0.6 cc. But after some time on a given dose the rate tended to fall again. The first period of heavy dosage resulted in greater stimulation than did the second.

The dog gained weight until the last period of administration, which terminated on February 20, and did not recover fully until two months later.

Blood Ca reached the high point of 18.59 mgm. on March 6, 13 days after the last dose, and progressively declined to 10.12 on July 7 except for one unexplained elevation to 13 mgm. on March 20.

The daily average urine volume decreased slightly during the 4th and 5th weeks (December 4 to December 18) but increased again during the 6th week (to December 25). Through the 7th and 8th weeks the foreperiod average was maintained. But during the 9th, 10th, 11th, 12th, 13th and 14th weeks, which correspond to the period of administration

begun on January 5, the daily average again decreased by about 50 cc. But with the 15th week (Feb. 20) began a great increase in urine output that persisted through the 32nd week, or up to June 25.



Figs. 7 and 8

The numbered black dashes in the lower line on the charts indicate periods of injection of viosterol. The same amount was injected daily over the number of days included, except as explained below. The numbers under the dashes refer to the following:

1. 0.5 cc. 100 X viosterol per kilogram daily.
2. 0.3 cc. 100 X viosterol per kilogram daily.
3. 0.6 cc. 100 X viosterol per kilogram daily.
4. 4.5 cc. 100 X viosterol per kilogram daily first day.  
6 cc. 100 X viosterol per kilogram daily second day.
5. 3.5 cc. 100 X viosterol per kilogram Feb. 16.  
5 cc. 100 X viosterol per kilogram Feb. 17.  
11.7 cc. 100 X viosterol per kilogram Feb. 18, 19, 20.

The upper interrupted graph gives the body weight. In Fig. 8 the daily Ca intake is shown as corresponding to the dates on the abscissa. The third tier shows the daily average Ca balance for the week corresponding to the dates. The black portion of the column shows the average total Ca output, the clear space the Ca retention, the entire column, Ca intake. In the 2nd, 4th and 9th weeks the intake is shown by the white line through the black columns.

A good positive Ca balance was maintained throughout except during the 4th and 9th weeks. In the latter case there was an extraordinarily high average daily output of 1.22 gms. of which 1.189 gms. was by the intestinal route. The proportion of urine Ca tended to increase slightly from 2-3 per cent during the foreperiod up to 6 per cent in the 12th week (January 29 to February 5); 16 per cent in the 13th week (to February 12); 30 per cent in the 14th (to February 19); and 50 per cent in the 15th (to February 26). The balance experiment was discontinued at this point, but the urine Ca continued to rise through the 24th week (to April 30) with a daily average at this point of 165 mg. The highest figure for any one day was 257 mg. on March 30. After April 29 the daily average output declined sharply to 69 mgs. during the 25th week, ranging between this figure and 59 mgs. until the end of the 28th week (to May 28), then decreasing progressively to the original level by the 33rd week (June 26).

It is significant that the period of increased urine Ca elimination coincides with the enormously increased urine volume. During this period there was, of course, increased water intake. But it would not be justifiable to attempt to state at present which of these is the primary factor.

A positive P balance was maintained throughout except during the 2nd and 4th weeks. It is probable that neither of these is of any significance since in both instances the high average was due to excessive output on one day of the week. However the proportion of urine P increased progressively from a daily average of approximately 50 per cent during the foreperiod to 78 per cent during the 15th week.

In one other experiment a study was made of the effect of a single large dose on urine excretion. Dog Number 45 (Table I) was given a dose of 4.75 cc. 10,000 X (8.6 kilos.), the equivalent of approximately 55.2 cc./kgm. of 100 X viosterol. A legitimate criticism of this experiment might be made in view of the short foreperiod. Nevertheless, the figures for this period are comparable to those obtained on other normal dogs. The injection was made at 9:00 a.m. on the third day (May 9, 1931) and the blood Ca and P followed at intervals for 7½ hours and daily thereafter. Another 1 cc. dose of viosterol was given on the 33rd day, June 4. The significant features are the enormous increase in urine Ca output and in urine volume. Urine P output was not significantly altered. This animal did not lose weight until after the 13th day, having gained slightly up to that time.

In general there was fairly close parallelism between urine volume and the content of Ca.

TABLE I

No. 45

Date	Vol.	Urine		Blood		Wt. kilo.	Viosterol dosage and remarks
		Ca	P	Ca	P		
	cc.	mg.	mg.	mg.	per 100 cc.		
5-7	910	19	341			8.62	
8	1200	37.5	480				
9 9:00 A.M.	1450	20	421	11.44	5.12	8.62	4.74 cc. 10000X
9:30				10.84	4.44		
10:30				10.40	5.51		
12:30 P.M.				10.40	6.45		
2:30				10.40	6.66		
4:30				10.40	4.77		
10	1500	38	329	11.18	6.07		
11	990	14.8	322	11.99	6.83		Mild diarrhea
12	1300	60	512	15.80	7.27		
13	1400	127	537	19.89	4.94		
14	2250	275	595	14.00	6.06		
15	970	217	500	15.75	4.79		
16	270	43	131	16.85	7.48		Poor appetite, diarr.
17	1200	77.5	318	16.75	4.15		
18	580	65.5	196	15.70	5.33	8.70	Livelier
19	2700	309	513	15.30	4.33		
20	2500	333	567	16.35	5.35	8.84	
21	1550	166	291	16.20	5.00		
22	1600	183	200	12.84	5.58		
23	1650	216	324	13.70	5.59		
24	2500	263	506	15.29	6.61		
25	500	64	169	14.88	5.06		
26	3500	331	610	15.05			
27	2500	240	460	16.31	6.66		
28	2500	374	606			8.00	
29	2500	367	416	21.60	5.63		
6-2	2000	274	768	17.21	4.57		
3	1000	160	478				
4	2240	282.5	892	16.37	6.95		1 cc. 10000 X
5	1250	178.5	278	15.82	3.69		
6	1530	197.5	241				
7	1550	263	486				
8	740	112	200				
9	1300	112	263				
10	1100		160				
11	1900		500	18.30	3.72	7.48	Poor appetite
14	2500		1030				
15	1600		540		4.44	7.48	



In one human subject, a man of 65 kilos. weight, a balance experiment was done with a foreperiod of one week, after which 15 injections of 0.5 cc. 10,000 X viosterol were made over 16 days. On the 11th day only was the blood Ca significantly increased. A good N balance was maintained throughout with no significant alterations. There was definite P retention during two weeks and still greater retention during the remaining two days of injection, but during the 3 day after period, progressively increasing elimination.

Ca elimination was well balanced during the foreperiod, and during the first two weeks. There appeared a definite increase in the urinary fraction, both relative and absolute, with progressive retention. The patient experienced no subjective symptoms at any time and maintained constant weight.

### DISCUSSION

In every experiment a significant increase in the resting, post-absorptive metabolic rate has been observed following viosterol administration. This invariable stimulation of the metabolic rate that occurs with any large dosage of irradiated ergosterol is difficult to explain at present. The response is immediate in most cases, but is sometimes delayed. Occasionally an animal has responded by a decreased metabolic rate after a decrease in the dose administered. The fact that the rate frequently decreases on continued administration might be interpreted as due to tolerance or to an altered state of metabolism accompanying toxicity.

The increased metabolic rate is apparently not to be correlated quantitatively with any single observed variable factor. Examination of the charts of rate and depth of respiration taken during each experiment does not indicate any regular increase in the respiratory rate but the depth was usually increased very definitely. Where the rate was increased it was not regularly correlated with a pronounced increase in metabolic rate. The greatest increase in oxygen intake was, with five exceptions, correlated with pronounced increase in depth of inspiration. The weight loss, which occurred even with increased food consumption, is also a difficult reaction to explain. In the later stages, of course, after toxic symptoms have developed, there is usually little or no food consumption.

Aside from the augmentation of the metabolic rate the Ca output is of special significance. When there is a relative increase in the urine fraction with no alteration of exchange it seems probable that this is due to increased absorption of Ca from the gut. But decreased ingestion with a

shift from feces to urine must be interpreted as the result of increased Ca mobilization. Where this phenomenon persists for weeks after administration has ceased, as in the case of Number 27, it must mean that the tissue deposits of Ca which we believe to occur are being eliminated by way of the kidney. Microscopic examination of the kidneys shows some injury to the tubular epithelium. It has been generally accepted that such injury alone will permit greater Ca loss so this factor may also be concerned. The results of analyses of the tissues of these animals for Ca and P will be reported in another paper.

While the effects on P excretion are less pronounced than on Ca, in general, it may be said that there is retention during mild administration, with a shift from feces to urine and perhaps some tendency to increased excretion in the after period.

In this connection it should be pointed out that the true after period cannot be determined since there is no method of determining when the ergosterol accumulated in the body is exhausted.

The great increase in urine volume accompanying increased urine Ca excretion, while not an invariable result, is nevertheless common enough to warrant attention. What is the primary import of this effect has not been determined. There was always increased water consumption, but whether this was a primary or secondary effect cannot be stated at present.

While Thöenes reports stimulation of nitrogen metabolism under ergosterol administration, neither our experiments nor those of Kern, Montgomery and Still (6), nor of Bauer, Marble and Claffin (1) confirm this finding. Our experiments are too inconclusive to warrant further discussion of this point at present. This question will be investigated further.

### CONCLUSIONS

1. Administration of large doses of irradiated ergosterol to normal dogs causes an increase in the metabolic rate that is not quantitatively related to any other observed factor.

2. During administration at a low level there is usually retention of Ca with a shift from feces to urine. On heavy or long-continued administration there is increased urine Ca elimination although a good balance may be maintained.

3. To a lesser extent the same holds for phosphorus excretion.

4. During the period of increased urine Ca elimination there is usually a great increase in urine volume.

5. There is no evidence of any effect on nitrogen metabolism.
6. It is believed that one of the early effects of heavy dosage with ergosterol is increased consumption of body fat.

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## THE CALCIFICATION OF TISSUES BY EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL\*

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THE deposition of calcium in various tissues after excessive dosage of irradiated ergosterol has been generally observed by those engaged in investigation of this problem, with the exception of Cartland, Speer, and Heyl (1) and Tavares (2). Duguid (3), Spies and Glover (4), Laas (5), Hoyle (6), Simmonet and Tauret (7), Huebner (8), Smith and Elvove, (9), Schoenholtz (10), Holtz and Von Brand (11), Herzenberg (12), Schiff (13), Comel (14), and Light, Miller, and Frey (19) have reported calcium deposition in various organs up to 100 times the normal content of such tissues.

In the course of investigations on the effects of viosterol on normal dogs, various tissues were analyzed for calcium and phosphorus content. The details of the experiments to which the animals were subjected are given in another paper (Reed, Thacker, Dillman and Welch, 15), but a summary is given in Table I, with the exception of Numbers 27 and 30, in which cases the procedure was varied as described below.

Number 27 received a total of 225.8 cc. of viosterol, 100 X, over 34 days, or 15.36 cc. per kilo. of body weight. This part of the experiment was terminated on September 15, 1930. On December 3 the injection of viosterol was resumed. During the next 80 days a total of 1004.4 cc. of viosterol was injected, or 62 cc. per kilogram of initial body weight. This dog was not killed until July 7, 1931, about 4 months after the last injection.

Number 30 received a total of 315.5 cc. over a period of 22 days, or 25.65 cc. per kilogram of original weight. This period terminated on September 15, 1930, and the dog was allowed to recover until February 8, 1931. Over the next 12 days a total of approximately 870 cc. of viosterol 100 X was

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injected, or 56.5 cc. per kilogram. The dog died on the 23rd day after administration was begun, or 11 days after the last injection.

The irradiated ergosterol was administered by intravenous injection, as viosterol 10000 X, but in all cases the dosage is calculated as cubic centimeters of 100 X per kilogram of original weight. The fifth column in the table indicates the number of days each animal survived after the first injection, regardless of whether it died from the toxic effects or was killed.

The tissues to be analyzed were weighed immediately, then dried at 105°C. for three days and again weighed into vitrosil crucibles, and ashed at 800°C. The ash was then dissolved in 10 cc. of 20 per cent trichloroacetic acid, and diluted to 25 cc. in a volumetric flask. Portions of this solution were used for calcium determination by the method of Shohl and Pedley (16) and for phosphorus by that of Fiske and Subbarow (17). The results as shown in Tables III and IV were so variable that it was deemed best to resort to a statistical treatment of the data. Accordingly, 13 apparently normal dogs were killed and the tissues analyzed. In addition, one dog, Number 31, received 2 cc. of corn oil daily over 19 days in order to rule out possible influences of the oil itself. This amount is far in excess of any volume of corn oil actually injected as viosterol. The figures for the tissues of Numbers 27 and 31 are not included in the statistical comparisons, although both are shown in the table. Not all tissues were included for some of the animals. This fact renders the statistical comparison somewhat less significant. Aside from the value of the comparative results, it is possible that a record of the tissue content of calcium and phosphorus in normal animals may be of some value to others.

Of the 12 tissues analyzed from 13 normal dogs, the mean Ca values ranged from 14.3 mgm. per 100 gms. of dried tissue in the spleen to 101.3 mgm. in the thyroid. The highest values for each tissue ranged from 25 mgm. in muscle (Numbers 69, 80) to 263 mgm. in the thyroid (Number 66). The lowest value found in any one tissue was 7 mgm. in the spleen (Numbers 69, 71). The greatest standard deviation was found in the thyroid.

The mean values for P content ranged from 152.3 mgm. in the skin to 1317 in the brain. The highest values for each tissue ranged from 34 mgm. in the skin (Number 74) to 1526 mgm. in the brain (Number 70). The largest standard deviation was found in the kidney.

Of the tissues of Number 31 that were examined, the Ca content was usually within the normal range for each tissue but generally well above the mean. However, in the left ventricle, muscle, and skin, the values were

well above the range for the normal group. The P content shows much the same variation, being markedly higher in the aorta than the mean of the normal group, but in all other tissues the P content was in the upper extreme of the range, except in the left ventricle. These differences are of no significance statistically.

In the dogs that had received irradiated ergosterol, every tissue examined showed a higher mean Ca content than in the control series, except Number 27. As will be seen on comparing Tables I, III and IV, there was no direct correlation between the calcium content and total amount of viosterol injected, the amount injected per kilogram of body weight, the number of days, or the size of individual injections. The variations in the viosterol series probably would have been less extreme if every animal had been treated in exactly the same way. But since the major investigation had to do with the mechanism of physiological action of viosterol, it was inevitable that the treatment would be varied. Furthermore, the progress of the experiments frequently conditioned the procedures. While no two animals *were* treated in exactly the same way, it is clear that wide

TABLE I

No.	Initial wt. kilos	Total cc. vosterol 100X	cc. 100 X viosterol/ kgm.	Days	No. doses	
5	10.4	520	50	62	18	Died
17	16	1498	93.6	73	42	Died
21	15	1143	76	43	16	Died
24	12	518.4	57.6	47	9	Killed
25	14.2	544.6	38.4	33	32	Killed
26	8.5	260.1	30.6	17	17	Died
29	11.7	1254.8	107.25	30	26	Died
30	12.5	*870	56.5			Died
32	11.4	305	26.75	23	17	Killed
33	11.4	340	28.95	33	18	Killed
35	12.2	184.6	15.13	29	29	Killed
45	8.6	575	67	35	2	Killed
108†	10.5	1700	161.9	9	6	Died
109‡	12.7	1750	137.8	8	8	Died
27	14.5	*				

\* Detailed discussion of the treatment of these two dogs is given in the text.

† Viosterol administered after a 10 day rest period following injection of 85 cc. of parathormone over 31 days.

‡ Viosterol administered after a 10 day rest period following injection of 94 cc. of parathormone over 18 days.

variations in tissue deposits of calcium and phosphorus would result, even if all the animals responded in the same way to a given treatment. However, it is apparent that there are wide variations in the individual animals' capacity to respond to viosterol administration, as has been pointed

TABLE II  
Calcium, mgm. per 100 gm.

Tissue	Normal mean a	Ergosterolized mean b	$E_{\Delta} = \sqrt{a^2 + b^2}$	$\Delta/E_{\Delta}$	Probability
Adrenal	37.6 ± 3.24	203.1 ± 81.9	81.9	2.02	0.04
Aorta	64.23 ± 6.84	175.3 ± 35.7	35.9	>3	<0.01
Brain	55.4 ± 14	125 ± 16.7	21.8	>3	<0.01
R. Ventricle	2.27 ± 4.43	149.6 ± 37.69	37.9	2.9	<0.01
L.V.	18.3 ± 1.64	220.4 ± 63	64	>3	<0.01
Kidney	55.6 ± 15.9	671.6 ± 236.3	236.8	2.6	<0.01
Liver	17.9 ± 1.22	122.8 ± 45.1	45.5	2.33	0.02
Lung	88.6 ± 13.2	338.4 ± 160.2	165	1.514	0.13
Muscle	17.8 ± 1.16	64.8 ± 13.3	13.4	>3	<0.01
Skin	23.6 ± 1.95	69.8 ± 16.1	16.2	2.85	<0.01
Spleen	14.3 ± 2.19	100.8 ± 21.26	21.4	>3	<0.01
Thyroid	101.3 ± 22.9	509.3 ± 144.66	147	2.77	<0.01

Phosphorus, mgm. per 100 gm.

Adrenal	595.8 ± 30	648 ± 109.18	113.3	<1	0.64
Aorta	272.2 ± 18.4	315.4 ± 13.1	28.07	<1	0.12
Brain	1317.3 ± 31.5	1018.3 ± 130	164	1.82	0.07
R.V.	708.1 ± 37.7	736.9 ± 42.36	50.6	<1	0.61
L.V.	767.3 ± 50.3	694.5 ± 49.2	70.4	1.34	0.27
Kidney	815.8 ± 74.3	994 ± 126.5	146.7	1.21	0.23
Liver	790 ± 24	681.5 ± 104.1	106.8	1.02	0.30
Lung	865 ± 53.3	977.6 ± 163.6	172	<1	0.51
Muscle	699.3 ± 43.6	536.7 ± 47.7	60.4	2.19	0.03
Skin	152.3 ± 37.1	280 ± 50.5	62.5	2.05	0.04
Spleen	925.1 ± 40.8	901.3 ± 59.4	72.12	<1	0.74
Thyroid	554 ± 57	712.6 ± 119	132	1.2	0.23

$E_{\Delta} = \sqrt{a^2 + b^2}$ ,  $a$  = standard deviation of normal series.

$b$  = standard deviation of experimental series.

$\Delta/E_{\Delta}$   $\Delta$  = difference between means of the two series

out by Thöenes (18). Another factor which might alter the statistical evaluation somewhat is the failure to include in the analysis all of the tissues for each dog in the experimental group.

There were individual animals in the experimental series with values well within the normal range. For example, Number 35 approximated the

mean values of the normal group in every tissue except in the skin, spleen and thyroid. Number 108 also was well within the normal range for the adrenal, aorta, kidney, and skin, but all other tissues showed high values.

Number 26 had the highest Ca content of all animals examined in the adrenal, brain and lung; but was third in order in the aorta, the right ventricle, left ventricle, skin, and spleen; and second in order in the kidney and liver, while in muscle the figure was only slightly higher than the highest figure for the normal group. Yet, from Table I, it is seen that this dog received next to the lowest total amount of viosterol and fourth in ascending order of amount per kilogram.

Number 30 showed the highest values for the right ventricle, left ventricle, kidney, liver, spleen, and thyroid and the second highest values for the lung and muscle, whereas for the aorta, brain, and skin the values were within the normal range.

In general, then, there is a tendency toward a comparable degree of Ca deposition in the majority of tissues of any one animal, but this tendency is by no means invariable, as any amount that will induce toxic symptoms induces wide variations.

In case of Number 35, it was believed that the viosterol dosage was near the threshold of toxicity for this dog, as judged by the response of the metabolic rate and urine Ca excretion, and the steady maintenance of body weight, as described in the paper previously referred to (15). The Ca content of all tissues in this dog closely approximated the mean values for the normal series except in the skin, spleen and thyroid.

The values obtained for Number 27 were comparable to the mean values for the corresponding tissues in the normal group, except in liver and muscle. In view of the fact that this dog lived approximately four months after the last viosterol administration, it seems probable that whatever excess Ca deposition occurred must have been removed during this period. Judging from the other animals, such deposition must have occurred at some time. It is not clear why the liver, particularly, and muscle to a lesser degree, retained such high contents.

In Table II is shown the statistical treatment for each tissue. The standard error (S.E.) was determined by the formula  $S.E. = S.D. / \sqrt{n}$ , where S.D. is the standard deviation. The figures in the fourth column were obtained by dividing the difference between the means of the two series by the factors in the third column. The last column represents the probability of normally distributed values that would have positive or negative deviations exceeding the standard deviation in the ratios shown



NORMAL Docs—Mgm. Ca per 100 gm. Dried Tissue

[illegible]

Mgm. P. per 100 gm. Dried Tissue

81	800	31	730	70	1526	78	1008	78	1094	74	1112	73	990	78	1143	73	939	74	344	78	1198	78	802
68	736	80	398	72	1445	69	994	70	979	78	1073	80	897	69	1108	78	920	72	296	73	1185	73	801
74	725	78	342	66	1410	80	920	73	967	70	1020	81	870	80	1042	74	839	73	234	65	1180	81	796
65	630	81	338	31	1407	81	829	71	946	73	1015	69	854	68	1013	71	810	81	206	84	994	31	794
78	627	71	329	68	1399	31	760	81	845	72	1007	65	800	67	946	81	769	31	201	80	953	74	791
66	618	73	314	80	1398	71	721	74	781	69	970	68	790	M	865	72	739	78	188	M	925	80	745
M	595	72	290	74	1364	M	708	M	767	71	962	M	790	72	842	69	724	M	152	70	913	68	586
70	584	M	272	M	1317	66	707	69	760	81	874	67	782	70	841	M	699	65	116	69	871	M	554
80	575	65	268	67	1304	68	668	62	725	M	815	66	767	66	801	80	686	80	110	68	869	71	530
72	549	70	248	65	1299	65	656	72	682	80	795	78	742	65	778	67	625	68	92	71	850	65	448
69	541	68	242	78	1280	73	734	31	676	31	764	70	733	74	765	66	591	66	86	72	820	66	448
73	489	66	218	69	1230	74	632	66	580	66	554	71	715	71	710	31	520	67	84	66	814	72	374
71	481	71	197	71	1215	72	631	69	555	67	445	72	669	73	702	65	520	70	80	67	798	67	338
67	390	69	182	81	1130	70	596	80	548	68	425	74	662	31	645	68	520	71	77	81	682	70	300
31	—	67	173	73	1125	67	581	65	513	65	354	31	512	81	555	70	409	69	73	31	—	69	243
Mean	595.8	272.2	1317.3	708.1	767.3	815.8	790.0	865	699.3	152.3	925.1	554.0											
S.D.	108.4	66.5	113.5	136.0	181.6	268.0	86.8	192.1	157.5	133.8	147.3	205.6											
S.E.	30.0	18.4	31.5	37.7	50.3	74.3	24.0	53.3	43.6	37.1	40.8	57											

S.D. Standard Deviation  
S.E. Standard Error



Mgm. P. per 100 gm. Dried Tissue

25	1137	32	473	33	1968	30	1035	109	1009	109	1705	5	1410	45	2440	26	784	30	761	5	1335	109	1190
19	936	26	446	21	1511	24	866	21	841	21	1650	21	1333	26	2156	5	756	21	446	26	1272	45	1079
26	767	24	349	26	1488	33	819	26	834	26	1503	45	893	24	1439	24	670	25	368	24	996	29	937
27	764	29	339	24	1241	21	775	45	822	5	1394	26	690	M	977	108	670	26	343	32	994	08	765
33	762	33	338	17	1220	27	754	33	797	24	1282	M	681	21	950	27	645	29	324	45	993	21	723
32	674	M	315	27	1192	25	747	32	788	M	994	27	644	17	882	21	638	M	280	27	986	M	712
M	648	35	273	108	1169	M	736	108	742	21	946	29	630	32	796	32	589	17	221	21	940	25	497
109	500	25	227	109	1164	26	729	M	694	108	941	109	601	5	793	33	570	24	208	M	901	35	388
108	216	30	215	M	1018	108	659	29	641	32	905	32	523	25	690	45	564	35	181	109	870	27	383
35	196	45	198	5	924	109	644	24	602	29	826	33	504	33	690	25	537	109	169	33	832	30	125
5	—	27	197	25	731	5	560	17	583	30	507	30	452	27	682	M	536	33	137	25	801	5	—
21	—	109	193	32	554	17	560	25	523	27	464	25	396	30	644	109	505	45	101	29	756	17	—
24	—	108	182	35	492	45	552	30	461	35	441	35	392	108	578	29	440	108	101	108	703	24	—
29	—	5	—	45	466	29	495	35	446	25	415	108	351	109	565	30	365	27	42	30	629	26	—
30	—	17	—	30	312	32	—	27	201	17	409	17	—	29	550	35	275	5	—	35	597	32	—
45	—	21	—	29	—	33	—	5	—	33	—	24	—	35	509	17	151	32	—	17	—	33	—
Mean	648	315.4	1018.3	736.9	694.5	994.0	681.5	977.6	536.7	280.0	901.3	712.6											
S.D.	309	43.5	471.3	.46.6	172.6	456.1	360.6	612.1	178.4	174.8	214.4	339.3											
S.E.	109.2	13.1	130.0	42.4	49.2	126.5	104.1	163.6	47.7	50.5	59.4	119											

S.D. Standard Deviation  
S.E. Standard Error

in the fourth column of Table II as determined from Fisher's table (20). According to this author, unless the factor  $\Delta/E_{\Delta}$  is 3 or more, the observed differences between the mean values are not significant statistically. By this criterion, only the aorta, brain, right ventricle, left ventricle, muscle, and spleen showed mean differences for Ca that are significant, although the probability is 1 per cent or less in the kidney, skin, and thyroid. Ranging from 2 to 4 per cent are the adrenal and liver, the lung alone showing a high percentage of probable variation beyond the standard deviation. However, it appears that only in case of the lung is there a probability of any great magnitude that the difference between the two series is the result of chance; here the probability is 0.13 or 13 chances in a hundred. In view of the fact that the experimental series does not represent homogeneous sampling even this figure does not entirely invalidate a conclusion that the experimental treatment was responsible for the definitely higher mean value.

It will be noted in Tables III and IV that in certain tissues the Ca content for a few animals is so far at variance with the range of the rest of the series as to be considered of a different magnitude. For example, the adrenal of No. 26 showed a value of 777, nearly three times as large as the next highest figure. These occasional variants necessarily increase the standard deviation and error. Excluding this one figure, the mean becomes 121, standard deviations,  $90.6 \pm 34.16$ ,  $\Delta/E_{\Delta}'$ , 2.4 and the probability is reduced from 4 per cent to 1 per cent.

However, there seems to be a fair probability that a larger number of animals in each series would show more significant differences, so that it seems to be a valid conclusion.

The mean reduction in the P content of the brain, according to Table II, results in a probability of 0.07 or 7 chances in 100 that the difference is due to chance. The probability for muscle and skin are 0.03 and 0.04 respectively or 3 and 4 chances in 100 that the differences are due to random sampling. In all other tissues the probabilities are so high that in the absence of a larger series it must be questioned whether irradiated ergosterol has influenced the P content.

In neither series was there any close correlation between the Ca and P content of a given tissue.

The significance of Ca deposition in the tissues cannot be evaluated at present. Microscopic examination showed degenerated cells in some tissues but this was not a constant finding except in the kidney. Here one gains the impression that cellular injury occurred first, in which case Ca dep-

osition probably occurred as a direct result of such injury. But this does not account for the findings in other tissues because of the infrequency of microscopic evidence of injury, even where extensive Ca deposition had occurred.

#### SUMMARY AND CONCLUSIONS

1. Analyses were made for Ca and P content of 12 tissues from 13 normal dogs and from 14 dogs that had received, by intravenous injection, toxic doses of irradiated ergosterol in the form of viosterol, 10000 X.

2. Statistical treatment of the data shows that the Ca content of any tissue may be significantly increased by viosterol administration, although wide variations may occur, certain tissues showing no increase.

3. The magnitude of the increase in Ca content is not correlated with the viosterol dosage but seems to depend on some undetermined individual factor or factors.

4. The P content, while widely variable among individual animals, was affected by viosterol administration in a much less constant manner, if at all.

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# VARIATION OF BASAL METABOLIC RATE PER UNIT SURFACE AREA WITH AGE

## II. THE PUBERTAL ACCELERATION

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THE variation of the basal metabolic rate per unit surface area with age during the pubertal period requires special definition.

Originally Du Bois (1) found the calories per square meter per hour of several prepubescent boys above the trend located by reported normals for preceding and subsequent years. On this basis he hypothesized a prepubertal rise in basal metabolism (2, 3). Subsequent to the establishment of puberty the same boys gave rates in agreement with the trend (4).

But Benedict and Talbot (5) observed no tendency in either sex toward a prepubescent divergence of the basal metabolic rate from its earlier rectilinear trend.

Yet the determinations of Benedict and Hendry (6) of the minimum metabolic rates of groups of sleeping girls of closely similar ages, supplemented by those of Benedict (7), plot its decline as following a fairly symmetrical sigmoid curve with its inflection in the fifteenth year, which is the earliest year for which all the members of a group have menstruated. The individual determinations of Bedale (8) on girls awake but still abed agree with these group determinations (9).

Similarly the trend of the means of the yearly averages of MacLeod (10) and of Blunt et al. (11) of the basal metabolic rate of girls under comparable clinical conditions, weighted with the number of subjects on which each is based, pursues a skew sigmoid curve with its inflection in the fifteenth year.

Further, Göttche (12) found the earlier gradual decrease in the basal metabolic rate for both sexes (recalculated for surface area (24): Table I, Chart 1) interrupted by a sharp prepubertal and pubescent increase, followed by a rapid post-pubertal recession. And Lax and Petényi (13) found the basal metabolic rate during the pubertal period to conform to the Du Bois curve with its prepubertal rise (1-4).

In a very large series of extremely accurate determinations, obtained by averaging the results of several duplicate tests which showed no downward



trend for each subject, Bierring (14) found an excess of relatively high basal metabolic rates during the fourteenth and fifteenth years in boys with manifest signs of puberty. The median trend of these data (Chart 2) shows a retardation of the decrease in the basal metabolic rate during the

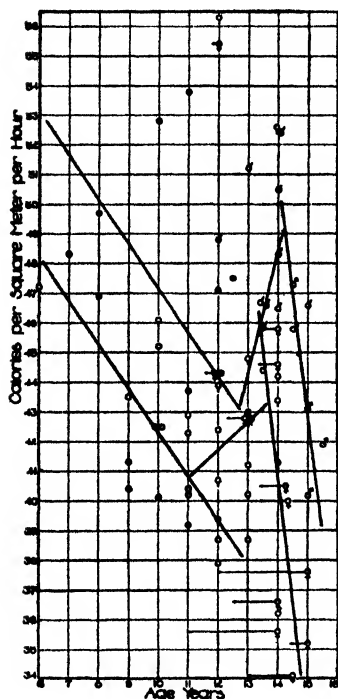


CHART 1

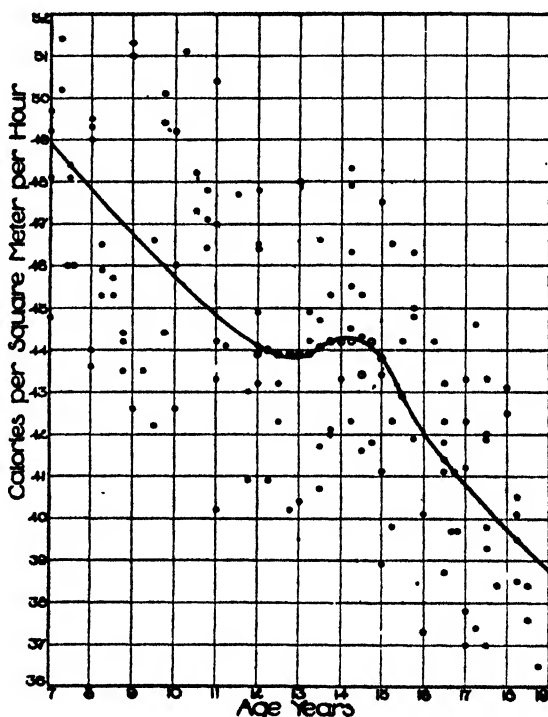


CHART 2

CHART 1. Basal metabolic rate of boys and girls (after data of Göttsche).  $\odot$  represents boys,  $\circ$  represents girls, prior to puberty.  $\sigma$  and  $\varphi$  represent respectively boys and girls subsequent to puberty.  $\sigma$  without its spear-head, and  $\varphi$  without its cross-bar, represent respectively boys and girls during pubescence.  $\sigma$  with its shaft projecting from the center of the circle and without the spear-head represents boys immediately before pubescence. The lines and arrow-heads to the left of  $\varphi$  point to the time of the first menstruation. The graphs represent the successive trends before, during, and after puberty. During the periods in which the graphs overlap separate standards would seem from these data to be indicated according to the pubertal status of the subject.

CHART 2. Basal metabolic rate of boys (after Bierring). Scatter diagram. Graph of median trend.  $\circ$  represents nine-point moving median.

thirteenth year, a distinct rise in the fourteenth year, and an accelerated decline subsequently.

By making successive determinations on a number of children of each sex at intervals during the pubertal period, Topper and Mulier (15) (re-

TABLE I  
CALORIES PER SQUARE METER OF BODY SURFACE PER HOUR DURING PUBERTY (AFTER GÖTTSCHE)

Sex	Age	Apubertal				Pubescent				Pubertal			
		Height	Weight	Area	Rate	Height	Weight	Area	Rate	Height	Weight	Area	Rate
Male	ys.	cm.	kg.	sq. m.	cal.	cm.	kg.	sq. m.	cal.	cm.	kg.	sq. m.	cal.
	6	86	11.5	0.52	61.4								
	7	114	22.2	0.82	48.3								
	8	131	28.1	1.02	46.9								
	9	119	22.2	0.86	49.7								
		128	25.1	0.95	40.4								
	10	130	24.7	0.96	42.5								
	11	126	21.6	0.88	52.8								
		137	30.4	1.08	43.7								
	12	123	20.2	0.86	53.8	147	39.3	1.27	48.8†				
	12½	124	25.3	0.94	44.3								
		133	27.1	1.00	47.1								
	13	135	26.1	1.00	47.5	162	33.0	1.27	51.2				
	13½					142	30.2	1.11	44.4				
	14					165	45.7	1.48	45.8				
						150	35.0	1.23	46.6				
						138	29.7	1.08	46.7				
						157	42.2	1.37	36.2				
						152	38.4	1.29	46.5				
	14½					142	31.9	1.13	48.3				
						149	36.0	1.24	50.5†				
						142	33.0	1.16	52.4				
										139	30.5	1.10	45.8
										141	39.5	1.24	47.3
	15					133	33.5	1.11	46.6				
										150	38.3	1.28	40.2
										159	51.5	1.51	43.1
	15½									160	48.8	1.49	41.9

Prepubertal.

TABLE I (continued)  
CALORIES PER SQUARE METER OF BODY SURFACE PER HOUR DURING PUBERTY (AFTER GÖTTSCHE)

Sex	Age	Apubertal				Pubescent				Pubertal			
		Height	Weight	Area	Rate	Height	Weight	Area	Rate	Height	Weight	Area	Rate
Female	ys	cm.	kg.	sq. m.	cal.	cm.	kg.	sq. m.	cal.	cm.	kg.	sq. m.	cal.
	3½	100	14.6	0.63	47.9								
	6	104	18.3	0.71	47.2								
	9	130	28.0	1.01	41.3								
		131	24.1	0.96	43.5								
	10	135	28.2	1.04	40.1	141	42.0	1.26	46.1				
		130	25.2	0.96	42.5								
		123	30.3	1.00	45.2								
	11	134	29.2	1.05	40.2	150	33.5	1.21	39.2				
		145	30.0	1.13	40.4	139	42.5	1.26	42.3				
		141	33.0	1.16	42.9								
	12	139	31.0	1.10	37.9	155	47.5	1.44	39.4				
		140	33.5	1.15	38.7	136	28.8	1.06	40.7	152	42.6	1.35	44.3
		138	31.8	1.10	42.4	149	39.5	1.29	43.9	139	29.3	1.07	55.4
	13					132	29.1	1.03	56.3				
		137	30.6	1.09	43.0	146	31.8	1.16	38.7	156	42.7	1.39	42.8
						139	31.0	1.10	40.2				
						161	38.5	1.36	41.2				
						145	35.8	1.21	42.7				
						144	31.4	1.14	42.8				
	14					134	35.5	1.14	44.8				
						139	38.2	1.21	40.8	150	49.8	1.43	35.6
						162	51.5	1.53	43.4	151	48.0	1.42	36.6
						150	34.8	1.23	44.2	154	41.3	1.34	44.6*
										150	44.2	1.36	45.8**
	14½									150	44.5	1.36	40.5**
	14½									154	42.7	1.36	40.0*
	15					148	35.5	1.23	52.6	157	59.0	1.59	34.1
										156	44.4	1.40	35.2
										170	56.3	1.65	37.6

\*, \*\* Same subjects respectively.

calculated for surface area (24): Table II, Charts 3, 4) demonstrated a definite increase in the basal metabolic rate immediately before and during pubescence, followed by a corresponding fall after puberty is established.

In a preliminary report Sandiford and Harrington (16) found the decrease in calories per square meter per hour for both boys and girls to be nearly, if not absolutely, a linear function of age. But when first degree

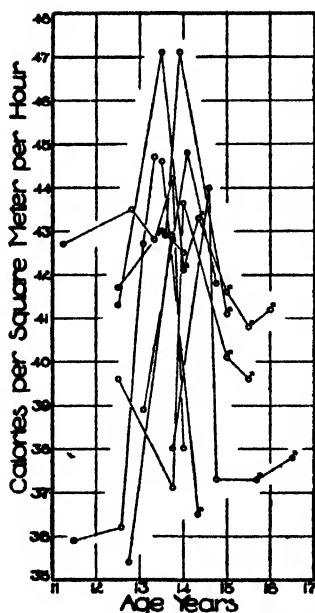


CHART 3

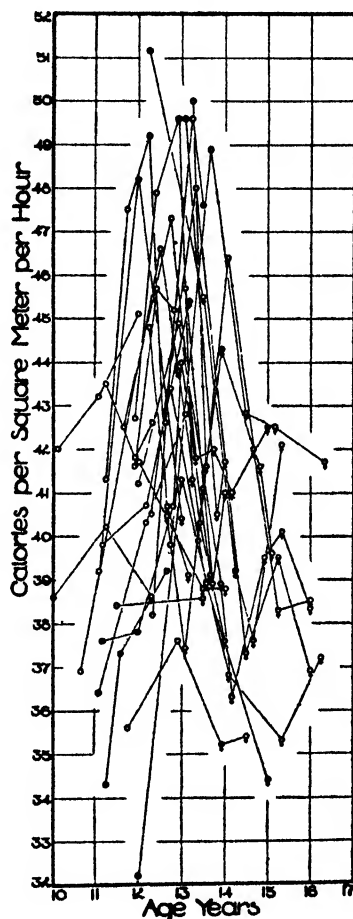


CHART 4

CHART 3. Basal metabolic rate of individual boys (after data of Topper and Mulier).  $\circ$  represents boys lacking signs of puberty.  $\circ$  with a horizontal line represents boys showing signs of puberty. A prepubertal rise occurs in each subject.

CHART 4. Basal metabolic rate of individual girls (after data of Topper and Mulier).  $\circ$  represents pre-pubertal girls.  $\bullet$  represents time of first menstruation.  $\circ$  with a vertical line represents post-pubertal girls. The prepubertal trend is upward, the post-pubertal trend downward. The individual peaks precede the first menstruation.

TABLE II  
CALORIES PER SQUARE METER OF BODY SURFACE PER HOUR DURING PUBERTY  
(after Topper and Muller)

Girls									
Age*	Height	Weight	Area	Rate	Age*	Height	Weight	Area	Rate
yrs.mos.	cm.	kg.	sq. m.	cal.	yrs.mos.	cm.	kg.	sq. m.	cal.
12:0	148	49.6	1.42	32.2	12:6	150	49.8	1.44	43.4
12:10					13:0				
13:0	154	48.9	1.45	41.3	13:7	155	56	1.54	38.9
14:1	158	56	1.56	36.8	15:0	159	58.5	1.60	34.4
15:4	160	57	1.58	35.3					
16:3	163	61	1.66	37.2	13:1	161	40.9	1.38	45.7
					13:3				
12:3	143	33	1.16	40.5	13:7	165	50.3	1.54	38.9
13:4	153	43	1.37	48.0	14:0	167	50.5	1.56	41.0
13:5									
14:6	153	46.6	1.42	37.3	12:0	157	79	1.80	41.7
15:3	154	46	1.41	39.0	13:0				
16:0	155	48.7	1.46	36.9	13:6	162	78	1.83	38.8
					14:0	162	78	1.83	38.8
12:0	142	41	1.26	41.2					
12:11	150	46	1.38	44.9	10:8	135	27	1.02	36.9
13:3					12:6	147	36	1.24	46.6
13:11	156	55	1.54	38.9	12:9				
14:2	157	58.6	1.59	36.3	12:11	149	35.5	1.24	43.3
14:11	158	60.3	1.61	39.5	13:5	150	40	1.30	40.3
15:4	160	63	1.66	40.1	14:0	153	41	1.33	37.6
16:0	160	63.4	1.66	38.4					
					11:9	145	49	1.38	35.6
13:4	148	41	1.30	39.9	12:11	160	67	1.70	37.6
14:1	155	44	1.40	46.4	13:1				
14:2					13:11	163	65.8	1.71	35.2
14:8	160	50	1.50	42.0	14:6	168	70	1.80	35.4
15:3	161	51	1.52	38.3					
16:0	161	52	1.53	38.5	11:3	138	32.4	1.13	41.3
					11:9	142	38	1.22	47.5
12:9	151	37	1.27	39.8	12:3	145	41	1.28	49.2
13:8	159	38.9	1.34	48.9	12:5				
13:10					12:8	150	43	1.35	40.5
14:6	162	39.8	1.38	42.8	13:1	150	46.5	1.39	37.4
15:2	163	42	1.41	42.5	13:6	150	47	1.40	41.1
16:4	165	46	1.47	41.7					
					10:0	139	45.4	1.30	38.6
12:3	146	35	1.21	51.2	11:3	142	53.2	1.41	40.2
13:0					12:1				
13:6	156	41	1.36	45.6	12:3	152	61.6	1.58	38.6
14:3	162	45	1.46	39.2					
14:8	168	50	1.55	37.6	11:6	143	57	1.47	38.4
15:4	163	55	1.58	42.1	12:11				
					13:6	157	70.5	1.71	38.6
11:8	146	33.5	1.19	42.5					
12:9	155	39.5	1.33	47.3	11:3	149	35	1.23	43.5
12:10					11:11	154	37.7	1.29	41.8
13:4	158	43.5	1.41	41.8	12:4	156	41	1.36	42.6
13:9	164	45.6	1.46	42.0					
14:2	165	49	1.53	41.0	11:1	139	29	1.07	39.2
15:0	166	50.5	1.55	42.5	12:0	149	34	1.21	48.2
					12:8				
					13:0	154	41	1.34	40.4

TABLE II(continued)

Girls					Boys				
Age*	Height	Weight	Area	Rate	Age*	Height	Weight	Area	Rate
yrs:mos.	cm.	kg.	sq. m.	cal.	yrs:mos.	cm.	kg.	sq. m.	cal.
11:3	148	48	1.40	34.3	12:6	138	32	1.12	41.3
11:7	150	49	1.42	37.3	13:6	145	35.8	1.21	47.1
12:8	157	50	1.48	39.2	14:0	146	35.2	1.21	42.1
					14:4	148	38	1.27	43.3
11:1	145	50.5	1.40	36.4	15:0	152	39.5	1.31	41.6
12:2	150	50	1.44	40.3					
13:1	156	50	1.47	42.8	13:9	165	69	1.76	38.0
					14:7	168	65.2	1.75	44.0
12:3	156	50	1.47	44.8	14:9	170	63	1.74	37.3
12:6					15:8	171	67.4	1.78	37.3
12:8	159	59	1.61	40.7	16:6	174	67.5	1.81	37.8
13:2	160	59	1.61	39.1					
					13:1	158	41	1.37	38.9
12:0	140	30.4	1.10	42.7	14:1	167	50	1.55	44.8
12:5	144	36.5	1.22	47.9	14:9	170	56	1.65	41.8
13:0	146	41	1.29	49.6	15:6	171	57	1.66	40.8
13:1					16:0	172	55	1.65	41.2
13:3	148	42	1.31	41.3					
					11:3	125	29	1.00	42.7
11:11	136	31.5	1.10	41.6	12:10	131	32	1.06	43.5
12:5	142	33	1.16	45.7	13:4	133	33	1.11	42.8
12:10	142	35	1.18	45.2	13:9	139	39	1.22	44.2
					14:0	141	44	1.30	38.0
12:4	135	32.5	1.10	38.2					
12:8	137.5	34	1.14	42.6	12:6	133	34	1.11	39.6
13:3	140	38	1.21	49.6	13:9	135	36	1.16	37.1
13:6	142	39.5	1.25	47.6	14:0	141	36.5	1.19	43.7
					15:0	149	41	1.30	40.1
13:0	152	31	1.17	49.6	15:6	151	45	1.37	39.6
13:1									
13:10	155	35	1.26	40.5	11:6	135	50	1.33	35.9
14:0	155	36	1.27	41.7	12:7	143	49	1.38	36.2
					13:1	145	50	1.40	42.7
10:1	130	27	.99	42.0	13:4	147	52	1.43	44.7
11:1	136	32	1.10	43.2					
12:0	142	37	1.20	45.1	12:6	119	25	.90	41.7
					13:6	129	28	1.01	43.0
11:2	125	31	1.03	37.6	14:0	130	29	1.03	42.5
12:0	134	32	1.09	37.8					
12:9	135	36	1.16	40.7	12:9	150	55	1.49	35.4
13:0	138	40	1.23	44.0	13:8	155	54	1.51	42.9
13:3									
13:8	141	43	1.28	39.1	13:7	132	31.5	1.07	42.9
					13:11	136	31	1.09	47.1
13:3	150	44.6	1.36	50.0	15:0	144	34	1.18	41.1
13:6									
13:7	152	44	1.37	41.6	13:6	148	42	1.30	44.6
13:11	153	45.6	1.40	44.3	14:4	158	49	1.48	36.5
14:10	157	49.5	1.47	41.6					
15:1	157	49	1.47	39.6					
11:2	137	30	1.08	39.8					
12:2	141	32.2	1.13	40.7					
13:2	148	39.5	1.28	45.4					

\* Black type figures signify the onset of menses in the female, and first observation of signs of puberty in the male.

equations are calculated by the method of least squares for the yearly means of the Boothby and Sandiford revision (17) of the Du Bois standards (Table III), the residuals for children show, in contrast to the haphazard scatter of the individual points to either side of the similar fitted straight lines for adults, a distinct tendency toward mounting to positive modes and tailing off negatively (Chart 5). The fitted straight lines for children are transposed with their slopes retained so as to render the re-

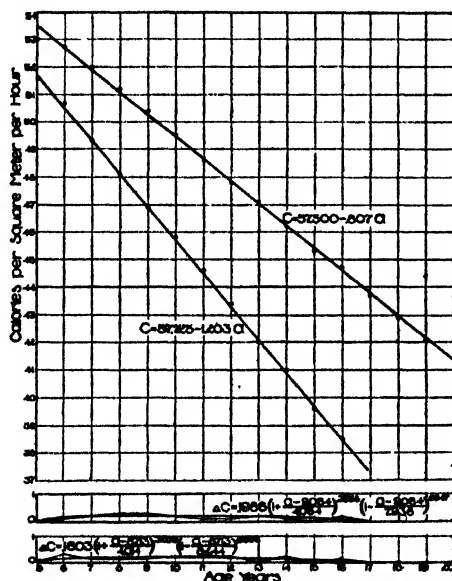


CHART 5. Basal metabolic rate (Boothby and Sandiford). Fitted straight lines. The individual points tend to mount above these lines medially and to fall below them terminally. If the lines were shifted so that all points would fall on or above them, the distances of the individual points above these lines would form the polygons shown in the lower details, to which modal curves are fitted. These curves would then represent cyclic increments. The shifted straight lines would represent the underlying decrease of the basal metabolic rate with age.

siduals positive or zero. Doubly limited, unimodal, skew curves equivalent to the polygons formed by the positive residuals (Chart 5) are calculated by the method of moments (18). The linear equations then represent the decrease of the basal metabolic rate with age, while the binomial equations represent cyclic increments (Table III).

An equation can as readily obscure as typify the actual mode of variation. The basal metabolism is the total exothermal product of the sum of chemical reactions proceeding in the post-absorptive resting organism. The variation of any factor which accelerates the basal metabolism, and

TABLE III  
CALORIES PER SQUARE METER OF BODY SURFACE PER HOUR (BOOTHBY AND SANDFORD)

Sex	Discontinuity and range	Equation*	Standard deviation
Male	yr.		cal.
	(4.5)-20.0	$C = 57.500 - 0.807a$	0.17
	(4.5)-19.0	$C = 57.400 - 0.807a + 0.1986 \left( 1 + \frac{a-9.064}{4.064} \right)^{0.2448} \left( 1 - \frac{a-9.064}{7.936} \right)^{0.8847}$	0.12
	(4.5)-20.6	$C = 58.44 - 0.817a$	0.24
	(4.5)-20.0	$C = 58.14e^{-0.0171a} + 0.4727 \left( 1 + \frac{a-9.405}{3.536} \right)^{0.1788} \left( 1 - \frac{a-9.405}{8.822} \right)^{0.4887}$	0.12
		$= 1 + 0.009719 \left( 1 + \frac{a-10.28}{4.408} \right)^{0.2658} \left( 1 - \frac{a-10.28}{7.950} \right)^{0.4878} \left\{ 58.14e^{-0.0171a} \right\}$	
	19.0-(79.5)	$C = 43.683 - 0.124a$	0.10
	20.0-(79.5)	$C = 44.61e^{-0.002a}$	0.07
	(4.5)-16.9	$C = 57.725 - 1.203a$	0.10
	(4.5)-16.0	$C = 57.625 - 1.203a + 0.1603 \left( 1 + \frac{a-8.713}{4.014} \right)^{0.3250} \left( 1 - \frac{a-8.713}{8.244} \right)^{0.8800}$	0.10
	(4.5)-17.2	$C = 59.68e^{-0.0271a}$	0.23
Female	(4.5)-17.0	$C = 59.50e^{-0.0272a} + 0.4644 \left( 1 + \frac{a-11.42}{8.390} \right)^{2.776} \left( 1 - \frac{a-11.42}{4.518} \right)^{1.486}$	0.11
		$= 1 + 0.01036 \left( 1 + \frac{a-11.57}{8.546} \right)^{2.507} \left( 1 - \frac{a-11.57}{4.362} \right)^{1.428} \left\{ 59.50e^{-0.0272a} \right\}$	
	16.0-(79.5)	$C = 38.786 - 0.0824a$	0.08
	17.0-(79.5)	$C = 38.98e^{-0.0084a}$	0.10

\* C = calories per square meter per hour, a = age in years, e = natural logarithmic base.





6). The exponential equations are recalculated to make the residuals positive or zero (Chart 6). Binomial equations for the resulting positive increments are calculated (Table III). The polygons and the fitted curves are plotted (Chart 6). These equations are recalculated to express their ordinates as divided by the ordinates of the exponential equations in order to represent the accelerative factor which elevates the basal metabolic rate above the base line of its decrease (Table III: Chart 6). Graphs of the percentile age distribution of the cyclic accelerative factor in each sex are drawn (Chart 7). These represent the pubertal metabolic acceleration.

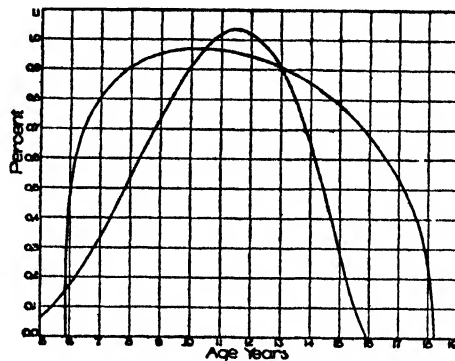


CHART 7. Pubertal metabolic acceleration. The accelerative factor in per cent is plotted against age. The round-peaked graph represents the male, the sharp-peaked the female.

The surface area to which the basal metabolism is referred itself increases at a variable rate. For puberty occurs during a period of growth acceleration. The adolescent growth cycle for weight has a range of from 10 or 11 to 20 years with its mode at  $12\frac{1}{2}$  years for females and 15 for males (20, 21). Smoothed curves of growth in surface area computed for children of each sex from the linear formula (22) or modifications of the Lissauer formula (23) based on it are similar to those of growth in weight prior to the pubertal inflection (5). Skew sigmoid curves plotting the values computed from yearly averages by the height-weight formula (24) pass through inflections at 12.3 years for females and 14.5 for males (25). The smoothed curve of all reported actual determinations of surface area plotted against age has its inflection at 14.2 years (26). Surface area during the growing period is related to weight as its two-thirds power times a constant (27). The surface area may then be computed from the weight series (20), and skew sigmoid curves of growth in surface area, and their

derived curves of velocity of growth in surface area, plotted (Chart 8). These latter curves mount gradually to a mode at 12.3 years for females, and, after remaining relatively level prior to 7 years, mount to a mode at 14.4 years for males, and decline toward zero toward 19 and 20 years respectively. The modes of the curves calculated for the pubertal metabolic acceleration occur at 11.6 years for females and 10.3 for males (Table III, Chart 7). These metabolic accelerative modes precede the growth accelerative modes. The modes of the pubertal metabolic acceleration would seem to coincide then rather with sexual maturation than with the modes of the adolescent growth cycle. The metabolic acceleration is

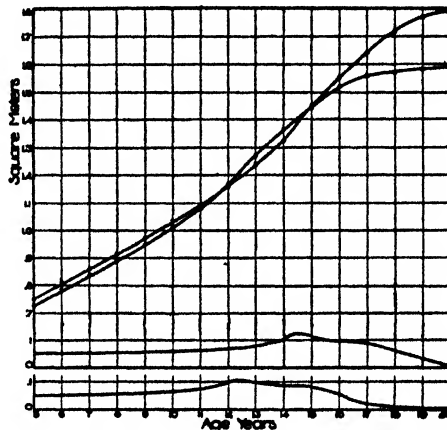


CHART 8. Growth in surface area (calculated from the weight series compiled by Davenport by means of the Boyd and Scammon formula  $S = 1.07 W^{0.478}$  in which  $S$  = body surface in square meters, and  $W$  = weight in kilos) and rate of growth in surface area. The sigmoid curves represent the total surface area of the body, the modal curves the rate of growth in surface area per year; in each case the upper curve represents the male, the lower the female.

in any case not a result or concomitant of growth. For if a temporary disproportion between the basal metabolism and the surface area arose during the growth cycle, the surface area would preponderate, and the already decreasing basal metabolic rate would be further diminished in relation to the surface area. Growth cannot therefore produce an appearance of a basal metabolic acceleration, but may tend to obscure this acceleration, and lessen or distort the apparent acceleration. The cyclic acceleration in the basal metabolic rate per unit surface area is not dependent on the adolescent growth cycle, but represents a specific acceleration of the basal metabolism associated with puberty.

## CONCLUSIONS

1. The basal metabolic rate during later childhood has been shown in numerous confirmatory investigations to give indications of a definite increase, or an irregularity in its decrease, related to puberty.

2. The variation of the basal metabolic rate per unit surface area with age during the pubertal period may therefore be analyzed as the mathematical resultant of a fundamental decrease of the basal metabolic rate with age, and a superimposed cyclic acceleration.

3. This cyclic acceleration of the basal metabolic rate is not a mere concomitant or effect of the adolescent growth cycle, but represents an independent pubertal metabolic acceleration.

Note: Full size blue prints or photostats of these charts can be obtained by writing to Dr. Curtis Bruen, 205 East 78th Street, New York City.

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## EFFECT OF PEARS, PEACHES, APRICOTS, AND DRIED SULFURED APRICOTS ON URINARY ACIDITY

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**I**N A previous paper (5) the effect of fresh Malaga grapes, several kinds of grape juice, grape concentrate, and Thompson seedless and Muscat seeded raisins on the urinary acidity was reported. The effect (6) of figs and smaller amounts of raisins also has been reported. In a subsequent paper (7) the comparative effect of tomato and orange juices has been given. These studies confirmed and considerably extended the earlier information obtained by Blatherwick (2) and Blatherwick and Long (3). In all cases, the above materials exerted a marked basic effect on the urinary acidity. Because of their present general importance in the diet, it also seemed desirable to ascertain the effect of daily ingestion of similar quantities of pears, peaches, apricots, and dried sulfured apricots.

In previous experiments, 1000 cubic centimeter portions of the juices and 1000 gram portions of the fresh fruit, or 300 gram portions of dried fruits were employed. In order that the results might be comparable, equivalent portions were used in the present experiments.

The tests were of twelve days' duration. As in the previous work, each experiment was divided into two consecutive periods. For the first five days each person received only the basal ration. During the next seven days each received, in addition, either 1000 grams of fresh Bartlet pears, or 1260 grams of canned apricots or peaches (including syrup of choice grade), or 300 grams of dried sulfured apricots containing 2140 parts per million of sulfur dioxide (except as stated later). During the last four days the dried apricots were cooked before serving. Three young men subjects were employed in each experiment, except in that with the dried sulfured apricots, where only two participated. All fruit was of choice commercial quality. The basal diet was the same as that described in a previous paper (5).

All analytical determinations were made in duplicate or triplicate, the average of closely agreeing duplicates only being reported. The methods of analysis were those described previously (5). In addition to the urine

analyses, determinations of the ash content, the alkalinity of the soluble ash, the alkalinity of the insoluble ash (1), and the organic acid content (8) were made upon composite samples of the fruits.

### RESULTS AND DISCUSSION

As is shown in Tables I to IV, with the basal diet, the pH of the urine generally reached equilibrium at 5.80 to 5.70. However, the equilibrium varied with the individual from pH 5.65 to 6.05. The average range compares well with the corresponding range observed in previous experiments (5, 6, 7). This is interesting, inasmuch as it indicates the extent to which basal reactions may be duplicated with a considerable number of apparently normal human beings. For example, in the various experiments in the general series of studies on urinary acidity, twenty-four different subjects have been used and the range of pH value on the basal diet has been within the above limits.

The effect of the fruits on the pH of the urine varied, ranging from an average change of about 0.7 pH units with pears to a minimum of 1.45 pH units' change with dried sulfured apricots. In all cases there was a marked increase of the pH of the urine following the addition of the fruits to the diet. Considering the pH of the urine on the last days of the experiment and that on the fourth or fifth day, the fresh pears produced an average increase of approximately 0.70 pH unit, the canned peaches approximately 0.85 pH unit, the canned apricots approximately 1.20 pH units, and the dried sulfured apricots an average increase of 1.45 pH units.

The relatively large increase of pH produced by the dried sulfured apricots in comparison with that produced by the unsulfured canned apricots is of interest. Apparently the inorganic sulfur added in the sulfuring process does not greatly reduce the basic effect of the apricot. This was emphasized by the fact that during the sixth, seventh, and eighth days of the diet, the dried apricots were eaten as such and contained about 2140 parts per million of sulfur dioxide, while during the ensuing or last four days of the diet, the dried fruit was cooked at a simmer for thirty minutes so that the final sulfur dioxide content was from 310 to 340 parts per million. On the average, about 85 per cent of the sulfur dioxide was thus removed, yet the additional increase of pH was only 0.35 to 0.45 pH units as compared to the total change of 1.45 pH units.

The average increase of pH of 1.20 units produced by the canned apricots is considerably greater than that of 0.85 pH unit produced by the

peaches; although both the values for the soluble and total alkalinities of the two fruits were quite similar. Likewise the increase of pH produced by the apricots is much greater than the respective ratios of either the soluble alkalinities or the total alkalinities (Table VI). If it is assumed that the

TABLE VI  
ASH CONTENT OF FRUIT

Material	Grams ash per 100 gms. fruit	Alkalinity of soluble ash cc. N/10 acid per 100 gms.	Alkalinity of insoluble ash cc. N/10 acid per 100 gms.	Total alkalinity cc. N/10 acid per 100 gms.
Pears	0.332	30.0	12.4	42.4
Peaches	0.533	48.0	11.0	59.0
Apricots	0.466	49.5	11.5	61.0
Dried apricots	3.293	294.0	77.0	371.0

pH is only finally changed by the basic constituents of the ash and that this change is approximately proportional to the alkalinity of the ash, then it appears that the fruits studied in this experiment do not produce a change in pH value strictly proportional to the alkalinity of their ash. However, the previous work (2, 5, 6, 7) has shown that the above assumption appears to generalize the magnitude of the changes of urinary pH produced by the other fruits studied. Further discussion of these relations will be given later. It is worthy of note that alkaline urines were produced by both the canned and dried sulfured apricots.

From the data in Tables I to IV showing the values for total acids and ammonia excreted daily, it is evident that there was a considerable decrease in their concentrations after the fruits were added to the basal diet. For the pears and peaches the concentrations were not finally reduced to such low values as in previous studies; but in both canned and dried sulfured apricots the total acids and ammonia excreted were greatly reduced by the fruit—the urines finally becoming alkaline.

The evaluation of the quantitative relationship between the excretion of acid in excess of fixed bases, as measured by determining the ammonia and titratable acid, and the carbon dioxide binding power of the blood plasma has been developed by Fitz and Van Slyke (4). In the previous study with grapes and grape products, this method of estimating the lowering of the alkaline reserve was employed. It was shown that the basal diet alone lowered the reserve and that the grapes and grape products assisted in securing a high alkaline reserve. Similar results have been secured



with figs, and tomato and orange juices. From a comparison of the data in Tables I to IV with those of the previous studies, it appears that the pears restored the average alkaline reserve to approximately what it had been at the beginning of the basal diet. Apparently the peaches increased the alkaline reserve somewhat above the normal as estimated from the data of the first day of the experiment. However, with both the canned and dried sulfured apricots, the alkaline reserve appeared to be very greatly increased above the normal for the individuals studied, and with approximately the same increase in both instances.

The organic acid contents of the pears, peaches, and the two kinds of apricots were determined by the method of Van Slyke and Palmer (8). The total amounts daily ingested, as cubic centimeters of one tenth normal acid, are given in Table V. The differences of the average daily organic acid titration of the urine with and without the above fruits added to the basal diet are given. From these values the percentage of the organic acids oxidized or otherwise destroyed in the body may be computed.

The average oxidation of the organic acids of the pears was approximately 94.9 per cent; of peaches 94.2 per cent; of canned apricots, 94.9 per cent, and of dried apricots, 94.6 per cent oxidation. For all four fruits the average was approximately 94.7 per cent oxidation. The percentage of organic acid oxidized was similar for all three fruits, although the total amount of organic acids ingested varied greatly according to the fruit used. For example, the organic acid content of the peaches was two and one-third times that of the pears.

For the purposes of comparison it may be recalled that, in the previous studies consisting of sixteen experiments with grapes and grape products, an average of 94.4 per cent of the organic acids expressed as tartaric acid was oxidized or destroyed. Averages of 97.5 per cent of the organic acids of the figs, 90.7 per cent of the organic acids of tomato juice, and 93.8 per cent of the organic acids of orange juice were oxidized. Apparently the oxidation or destruction of the organic acids by the body proceeded to approximately the same extent for the fruit materials investigated. This is particularly true of grapes and grape products, pears, peaches, canned apricots, and dried sulfured apricots.

#### SUMMARY

Experiments with men subjects on a basal diet and on the same basal supplemented by pears, peaches, apricots, and dried sulfured apricots are reported. The following results were observed when the fruits were added to the basal ration.

1. An average increase of the urinary reaction of approximately 0.70 pH unit was produced by 1000 grams fresh Bartlet pears; an average of 0.85 pH unit by 1260 grams of canned peaches and by an equal quantity of canned apricots, an average increase of 1.20 pH units. An average increase of 1.45 pH units was produced by 300 grams of dried sulfured apricots. Both kinds of apricots produced alkaline urines.

2. Corresponding decreases in both the ammonia excreted and in the total acidity were noted. The average changes produced by both kinds of apricots were considerably larger than those resulting from either the peaches or pears.

3. There was an increase of the alkaline reserve, estimated according to the method of Fitz and Van Slyke, equal to or above the normal for each subject. This increase was very marked for both kinds of apricots.

4. There appeared to be a correlation between the alkalinity of the ash and the reaction of the urine in the case of pears and peaches. A more basic reaction was associated with a higher ratio of soluble alkalinity to insoluble alkalinity of the ash. Compared with the peaches, the apricots appeared to produce an even more basic reaction with approximately the same ratio of soluble alkalinity to insoluble alkalinity.

5. A slight increase occurred in the organic acids excreted when pears, peaches, or the above two kinds of apricots were added to the basal ration.

6. The average percentage oxidations of the organic acids of the pears, peaches, and the two kinds of apricots were similar, averaging 94.7 per cent.

7. Apparently the added inorganic sulfur in the apricots does not markedly reduce the basic effect of the apricot on urinary acidity.

The writer wishes to express his appreciation of the interest and advice of Dr. W. V. Cruess, upon whose suggestion this study was initiated. The full co-operation of the men taking a part in the diets is gratefully acknowledged.

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TABLE I  
EFFECT OF FRESH PEARS ON COMPOSITION OF URINE

Days*	Subject S					Subject D					Subject M				
	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N
1	cc. 870	6.35	cc. 540	cc. 85	mg. 85	cc. 1270	6.10	cc. 555	cc. 175	mg. 85	cc. 880	6.35	cc. 538	cc. 134	mg. 86
2	1100	6.15	537	178	96	1150	6.25	518	221	121	1010	6.20	623	164	96
3	1280	6.00	567	177	116	1460	6.20	584	219	123	800	5.80	605	148	112
4	1050	5.85	502	162	153	1210	5.90	500	214	167	790	5.75	560	160	162
5	1050	5.90	526	145	137	1070	5.85	530	211	170	780	5.80	527	146	146
6	1330	6.20	553	194	149	1140	6.05	551	176	136	780	6.00	527	156	147
7	1460	6.45	584	169	143	1140	6.15	596	204	122	760	6.00	601	175	125
8	1040	6.55	610	164	95	1040	6.20	611	200	138	930	6.20	680	188	126
9	1120	6.75	675	95	94	1020	6.30	651	168	127	840	6.30	620	179	103
10	1580	6.75	616	104	108	1080	6.25	588	160	106	900	6.35	594	150	102
11	1860	6.75	676	86	117	1170	6.35	588	158	85	1050	6.40	660	133	92
12	1500	6.75	606	72	90	1070	6.40	525	129	71	980	6.40	599	106	84

\* Days, 1-5 inclusive, basal diet. Days, 6-12 inclusive, basal + 1000 grams fresh pear.

TABLE II  
EFFECT OF CANNED PEACHES ON COMPOSITION OF URINE

Days*	Subject A					Subject B					Subject D				
	Volume	pH	0.1-N organic acid	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acid	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acid	0.1-N titratable acidity	Ammonia N
1	cc. 880	6.45	cc. 740	cc. 123	mg. 173	cc. 850	6.00	cc. 530	cc. 192	mg. 119	cc. 750	6.15	cc. 378	cc. 106	mg. 126
2	1100	6.25	725	105	185	1210	6.00	620	220	203	880	6.00	503	148	148
3	860	5.70	675	172	187	860	5.65	590	238	272	840	5.90	492	215	188
4	1120	5.70	520	197	219	750	5.70	565	246	252	900	5.70	486	202	176
5	1220	5.65	573	181	206	1240	5.70	610	229	261	870	5.65	467	195	171
6	1080	5.75	674	222	181	1000	5.95	672	162	196	660	5.75	512	172	166
7	2000	6.30	650	132	168	1730	6.10	660	125	145	910	6.10	523	130	153
8	1670	6.35	855	134	164	1580	6.10	770	158	177	1090	6.10	654	153	152
9	1320	6.40	713	132	148	1270	6.10	684	153	178	960	6.20	590	128	135
10	2120	6.40	800	67	89	1750	6.25	744	84	121	910	6.25	742	109	115
11	1700	6.45	857	75	95	1670	6.35	808	94	140	910	6.40	810	109	102
12	1840	6.50	885	63	77	1620	6.45	837	78	91	930	6.60	876	82	91

\* Days, 1-5, inclusive, basal diet. Days, 6-12, inclusive, basal+1260 grams canned peaches and juice.

TABLE III  
EFFECT OF CANNED APRICOTS ON COMPOSITION OF URINE

Day*	Subject C					Subject O					Subject S				
	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N
1	cc.	6.20	cc.	cc.	mg.	cc.	6.10	cc.	cc.	mg.	cc.	6.35	cc.	cc.	mg.
2	980	6.20	395	62	127	860	6.15	495	112	193	1350	6.25	567	43	151
3	1200	5.90	625	60	135	760	6.05	434	94	171	960	6.00	618	99	188
4	810	5.90	541	195	258	810	6.00	590	146	182	1070	6.00	643	244	240
5	1330	5.90	468	187	262	1030	6.00	530	165	202	890	6.05	419	244	206
6	1040	5.90	432	203	263	690	6.00	527	159	193	880	6.15	450	257	222
7	980	6.30	451	110	247	1280	6.65	548	159	179	850	6.85	530	85	185
8	490	6.65	482	63	144	1110	6.75	591	142	170	1220	6.80	595	93	169
9	1790	7.10	516	72b	75	1650	6.85	731	40b	116	1680	7.10	692	27b	94
10	1820	7.10	828	77b	76	1550	6.80	670	81b	108	1370	7.10	695	66b	96
11	1550	7.15	861	81b	65	1700	7.10	758	75b	95	770	7.10	812	75b	86
12	1530	7.20	842	86b	64	1210	7.15	842	76b	85	1250	7.10	828	83b	70
	1580		893	77b	66	1370		814	82b	77	1390	7.15	857	86b	68

\* Days, 1-5 inclusive, basal diet. Days, 6-12 inclusive, basal + 1260 grams canned apricots and juice. b = denotes basic urines.

TABLE IV  
EFFECT OF DRIED SULFURED APRICOTS ON COMPOSITION OF URINE

Days*	Subject H					Subject T				
	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N	Volume	pH	0.1-N organic acids	0.1-N titratable acidity	Ammonia N
1	cc.		cc.	cc.	mg.	cc.		cc.	cc.	mg.
2	1080	6.00	462	151	121	660	6.40	465	122	129
3	850	5.95	588	172	167	960	5.95	610	173	161
4	870	6.00	535	174	195	990	6.05	594	171	193
5	890	5.90	431	199	199	1040	5.70	495	239	204
6	1460	5.70	494	187	204	850	5.65	492	232	214
7	730	5.75	461	170	204	720	5.65	504	166	202
8	1080	6.60	707	58	91	600	6.45	508	77	109
9	1180	6.70	854	28b	83	970	6.75	748	36b	81
10	1340	6.75	828	32b	75	1080	6.75	758	43b	91
11	900	6.85	760	65b	63	920	6.80	762	55b	77
12	820	6.85	780	66b	69	640	6.85	723	42b	72
	1020	7.15	823	69b	57	880	7.10	811	58b	62

\* Days, 1-5 inclusive, basal diet. Days, 6-12 inclusive, basal+300 grams dried sulfured apricots. b denotes basic urines.

TABLE V  
ORGANIC ACIDS INGESTED AND OXIDIZED  
(As cc. N/10 HCl)

	Pears			Peaches			Apricots			Dried Apricots	
	S	D	M	A	B	D	C	O	S	H	T
Average daily organic acid titration for basal diet period.....	534	537	571	647	583	465	492	515	539	502	531
Average daily organic acid titration for basal diet plus fruit.....	617	587	611	776	739	672	696	708	715	745	688
Difference.....	83	50	40	129	156	207	204	193	176	243	157
Organic acids ingested daily from fruit.....	1140	1140	1140	2840	2840	2840	3780	3780	3780	3690	3690
Per cent oxidation of organic acids.....	92.8	95.6	96.5	95.5	94.5	92.7	94.6	94.9	95.3	93.4	95.7



## THE IODINE CONTENT OF HENS' EGGS AS AFFECTED BY THE RATION\*

By

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THE increasing use of eggs in the human dietary necessitates a more complete knowledge of the variation in the amounts of the different essential components of the egg. Both the poultryman and the consumer are interested in eggs possessing a maximum nutritive value. The poultryman is also devoting his efforts to the production of eggs which contain the maximum amounts of the essential elements and compounds required for the development of strong, sturdy chicks.

A great deal of work has been done on the variations in the amounts of the organic entities of eggs (1). As a result of these investigations it has been definitely established that the vitamin A and vitamin D content of eggs can be greatly influenced by the ration of the bird. Work on the variation in the amounts of the minor inorganic elements in the egg has been given but little consideration. Elvehjem, Kemmerer, Hart, and Halpin (2) reported that neither the iron nor the copper content of eggs can be increased by feeding additional iron or copper. Unpublished work at this Station confirms these results for iron.

The iodine content of eggs, like that of other natural food products, appears to be variable. Forbes and co-workers (3) found traces of iodine in eggs. Hercus and Roberts (4) were able to increase the iodine content of eggs by iodine feeding. Simpson and Strand (5) increased the iodine value of eggs from 0.018 mg. to 0.754 mg. per 100 gm. of shell-free egg, by progressively increasing the dose of potassium iodide to 8 mg. daily over a period of four months. Within two weeks after the iodine feeding was discontinued, the iodine content of the egg dropped to 0.08 mg. per 100 gram of shell-free egg.

The common use of eggs in the human dietary, especially for infants and invalids, and the rather extensive use of iodine in the treatment of endemic goiter, make it desirable that we have further information on the

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extent to which the iodine content of eggs is affected by the iodine intake of the bird.

### EXPERIMENTAL

The initial experiment was in the nature of a preliminary trial to determine the comparative effects of different sources of iodine. Dried kelp, generally recognized as a potent source of iodine, fish meal, and potassium iodide were used. Chemical analysis of the kelp and fish meal used in this experiment showed that they contained 0.135 and 0.00124 per cent iodine, respectively.

Forty White Leghorn hens were divided into four lots of ten birds each and confined indoors. All lots received a basal ration of ground yellow corn 50, ground wheat 15, wheat bran 10, meat scraps 7.5, dried skim milk 5, alfalfa-leaf meal 5, bone meal 2, fine oyster shells 4, cod-liver oil 1, and salt 0.5. The birds were trapnested and eggs from individual hens were analyzed for their iodine content. After sufficient iodine determinations had been made, while the birds were on the basal ration, the rations were modified as follows:

Lot 1. Continued on basal ration.

Lot 2. Basal ration plus potassium iodide.

Lot 3. Basal ration plus 2 per cent kelp.

Lot 4. Basal ration in which meat scraps were replaced by an equivalent amount of Menhaden fish meal protein.

The quantity of potassium iodide added in Lot 2 was such as to make the iodine content of the ration comparable with that fed Lot 3 (kelp). All rations were fed *ad libitum*—no attempt was made to keep feed consumption records. The eggs from individual hens in the different lots were analyzed for iodine by a modification of the Kendall (6) method. While the regular method gave good results in the analysis of feed samples, it was found necessary to introduce certain minor modifications, in order to obtain satisfactory results in the analysis of eggs. An approximate 30 gram sample of egg was used. The method was checked frequently by the recovery of known amounts of iodine in the form of potassium iodide added to egg samples. Of 57 such determinations an average of 96.2 per cent recovery of iodine was obtained.

The results summarized in Table I show that the iodine content of eggs can be increased by increasing the iodine intake of the bird. The increase in iodine value was more or less proportional to the amount of iodine in the ration.

The results suggested that a more comprehensive study of the problem was desirable. The iodine-bearing substances had previously been mixed with the basal ration and fed *ad libitum*, with no check or information on the actual amount of iodine consumed per bird. Accordingly, in the next experiment, the birds were individually fed known amounts of iodine daily.

TABLE I  
IODINE CONTENT OF EGGS  
(Results expressed in parts per million of fresh, shell-free egg)

Average of all eggs before feeding iodine	Lot 1 Basal ration	Lot 2 Basal ration plus KI	Lot 3 Basal ration plus kelp	Lot 4 Basal ration with fish meal
0.56	0.57	8.25	7.81	0.77

Eighteen White Leghorn pullets were confined in individual cages, provided with wire floors, and fed the same basal ration as in the first trial. Individual egg records were kept and all eggs properly marked for identification. Eggs were saved for analysis after the birds had been on the experimental ration for seven days. After sufficient eggs had been saved for analytical purposes, the birds were divided into 3 lots of six birds each. The same basal ration was fed to all lots. In addition Lot 1 received dried kelp; Lot 2 iodized linseed meal<sup>1</sup> and Lot 3 potassium iodide evaporated on dextrin. These products were fed in gelatin capsules in such amounts that each bird received 2 mg. of iodine daily.

After the birds had been on these rations for four weeks the kelp feeding in Lot 1 was discontinued, and the amount of iodine in the form of iodized linseed meal and potassium iodide was increased to 5 mg. daily, in the case of Lots 2 and 3.

Iodine determinations were made on composite homogeneous egg samples from individual birds covering a period of one week. All determinations were made in duplicate or triplicate. The method was checked each day by recovery of known amounts of iodine added to samples of egg. Analyses were made on all eggs produced during the week prior to supplemental iodine feeding, and on those laid during the second and fourth weeks of periods 2 and 3, when the birds received 2 and 5 mg. of iodine daily, respectively. The results are recorded in Table II.

<sup>1</sup> We are indebted to Dr. G. M. Karns, of the Mellon Institute of Industrial Research, Pittsburgh, Pa., for the iodized linseed meal.

TABLE II  
THE EFFECT OF IODINE INTAKE ON THE IODINE CONTENT OF EGGS  
(Results expressed in parts per million of shell-free fresh egg)

Lot No.	Hen No.	Weekly periods									
		1	2	3	4	5	6	7	8	9	10
I		Basal ration		2 mg. iodine fed daily Kelp				No supplementary iodine feeding (Basal ration only)			
	1		#		6.42		7.80		0.66		0.25
	3		#		7.20		7.20		0.69		0.25
	4		#		5.14		10.42		0.30		*
	6		#		*		4.94		0.78		*
	7		#		4.68		*		0.36		0.25
	16		*		*		*		*		*
	Av.				5.86		7.59		0.55		0.25
II		Basal ration		2 mg. iodine fed daily Iodized linseed meal				5 mg. iodine fed daily Iodized linseed meal			
	8		#		6.34		4.22		*		*
	9		#		8.31		8.01		17.17		14.74
	11		#		6.74		11.40		16.57		19.97
	12		#		3.37		6.00		11.22		11.11
	13		#		5.02		7.88		9.74		10.60
	15		#		5.54		5.85		13.05		15.17
	Av.				5.89		7.37		13.55		14.31
III		Basal ration		2 mg. iodine fed daily potassium iodide				5 mg. iodine fed daily potassium iodide			
	5		#		5.62		7.62		12.74		14.85
	10		#		4.80		6.60		11.88		12.51
	14		*		*		*		*		*
	17		#		7.42		7.54		13.37		23.02
	18		#		6.80		9.34		14.80		20.77
	20		#		6.88		12.94		15.14		13.22
	Av.				6.30		8.80		13.58		16.87

# Approximately 0.1 part per million.

\* No eggs laid during week.

The data show conclusively that the iodine content of the egg is dependent upon and directly related to the iodine intake of the bird. These findings are in agreement with those of Hercus and Roberts (4) and Simpson and Strand (5). The per cent of iodine in the egg rises rapidly after iodine feeding, but apparently does not reach its maximum value until sometime after 2 weeks of supplemental feeding. The decline in iodine content is equally rapid after cessation of its feeding, but, like the increase, it required more than 2 weeks to reach again a minimum value.

The percentage of iodine in the egg was independent of the source of iodine when equivalent amounts of this element were fed. Apparently iodine in organic combination, as in kelp, is utilized no more efficiently than in the inorganic state. Eggs produced on the unsupplemented basal ration contained approximately 0.1 part of iodine per million. This amount, in all probability, was chiefly derived from the cod-liver oil in the ration. The feeding of 2 mg. of iodine daily, either as kelp, iodized linseed meal, or potassium iodide, increased the iodine content of the egg approximately 75 times. When the level of iodized linseed meal and potassium iodide was increased to 5 mg. of iodine daily, the iodine percentage of the egg was increased about 150 times.

No attempt was made to study the effects of an increased iodine content of the egg on hatchability, or the livability of chicks hatched from such eggs.

Whether eggs of a known iodine content have a particular place in human nutrition probably depends upon whether the iodine requirements of the people of a given locality are provided from other sources. It is common knowledge that the natural foods and drinking water of certain sections are deficient in iodine, and that some form of medication must be resorted to in order to prevent and control endemic goitre. How this can be best accomplished remains an open question. It would seem that medication through the use of natural foods, whose iodine content has been raised by administering the element to the animal, bird, or plant producing them, as suggested by Weston (7), Maurer (8), and Zickgraf (9) would in general be less hazardous and more desirable than by other means.

#### SUMMARY

1. The iodine content of hens' eggs varies directly with the amount of this element in the ration of the bird.
2. The feeding of 2 and 5 mg. of iodine daily per bird, in the form of

dried kelp, iodized linseed meal, or potassium iodide, increased the iodine content of the eggs approximately 75 and 150 times, respectively.

3. The percentage of iodine in eggs immediately decreases upon the discontinuance of iodine feeding.

4. The amount of iodine in eggs is independent of the form in which it is fed to the birds.

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## THE COMPARATIVE ANTIRACHITIC EFFICIENCY OF IRRADIATED ERGOSTEROL, IRRADIATED YEAST, AND COD LIVER OIL FOR THE CHICKEN\*

By

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THE effectiveness of cod liver oil and ultraviolet rays as prophylactic agents against rickets in the chicken has been definitely established. That irradiated preparations such as irradiated ergosterol and yeast are not so effective in the control of rickets in the chick as is cod liver oil when equivalent rat units of vitamin D are fed has also been reported by several investigators.

Mussehl and Ackerson (1) in 1930 reported that amounts of irradiated yeast and irradiated ergosterol theoretically equivalent to 10 and 50 per cent of cod liver oil, respectively, did not prevent leg weakness. At about the same time Massengale and Mussmeier (2) found that it was necessary to administer the equivalent of 200 per cent cod liver oil in the form of activated ergosterol to produce the effects of 2 per cent of cod liver oil. Hess and Supplee (3) and Russell and Klein (4) observed that it required more units of vitamin D in the form of irradiated ergosterol than in the form of cod liver oil to produce normal calcification in the chick.

Recently Steenbock, Kletzien, and Halpin (5) reported that it required from 40 to 120 per cent cod liver oil equivalence as irradiated ergosterol and from 7.5 to 60 per cent cod liver oil equivalence as irradiated yeast to produce the same degree of bone formation as 1 per cent of cod liver oil of average potency. The same investigators found no difference in the antirachitic efficiency or the degree of toxicity of irradiated ergosterol fed

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† Acetol Products Fellow. This investigation was conducted under a fellowship grant from Acetol Products, Inc., New York City.

in corn oil or in cod liver oil and accordingly concluded that vitamin D produced by ordinary irradiation of ergosterol with a quartz mercury vapor lamp is a different substance from that found in cod liver oil.

In our series of experiments, started in the fall of 1928, we attempted to compare the effectiveness of vitamin D in the form of irradiated ergosterol and irradiated yeast with ultraviolet light and cod liver oil and to determine, if possible, the reason for the difference in efficiency of the irradiated sterol and the natural vitamin D of cod liver oil.

### EXPERIMENTAL

Early in the investigation it was realized that irradiated ergosterol dissolved in linseed oil was not as effective in the treatment of rickets in chicks as in rats. It was also realized that in order to obtain an absolute comparison between the efficiency of cod liver oil and irradiated ergosterol and yeast the products would have to be fed on a known unit basis. Cod liver oil may vary as much as 100 per cent in vitamin D potency, whereas the activity of irradiated ergosterol or yeast may vary several thousand per cent. Accordingly, it is fundamental that the potency of the products be known and that the comparisons be made on a minimum unit protective basis if we are to draw intelligent conclusions.

In all our work the vitamin D potency of the cod liver oils, irradiated yeast, and solutions of irradiated ergosterol were determined on rats in our laboratory according to the Wisconsin Alumni Research Foundation procedure, and the activity expressed in Steenbock units.

The first experiments were of the prophylactic and curative type. The prophylactic series consisted of 5 lots, of White Leghorn chicks each, started on their respective rations when day-old. In the curative series, chicks which showed distinct symptoms of leg weakness at 7 weeks, while on the control ration, were divided into 5 lots of 18 birds each. All lots received a ration of ground yellow corn 52, ground wheat 20, wheat bran 5, dried buttermilk 20, calcium carbonate 2, and sodium chloride 1. This ration analyzed 1.02 per cent calcium and 0.45 per cent phosphorus. Varying amounts of irradiated ergosterol dissolved in linseed oil were incorporated in the rations of the lots in the prophylactic series, whereas in the curative trials definite amounts of the sterol in oil were fed in gelatin capsules daily. A negative control lot and a lot exposed to ultra violet light were included in both series.

The chicks in the prophylactic series were on experiment for 8 weeks, when 10 representative birds from each lot were killed for blood and bone

ash analysis. In the curative series, one-half of the chicks in each lot were killed after 10 days of supplemental feeding and the remainder at 19 days. The results of both series are recorded in Table I.

It required between 40 and 250 rat units of vitamin D per 100 gm. of basal ration as irradiated ergosterol to prevent leg weakness. On the other

TABLE I  
THE ANTIRACHITIC EFFICIENCY OF IRRADIATED ERGOSTEROL IN LINSEED OIL FOR CHICKS  
Prophylactic Trial

Ration supplements	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibia
			Ca. per 100 cc. serum	P. per 100 cc. serum	
		gm.	mg.	mg.	per cent
None (control) . . . . .	0	301	8.7	5.6	42.9
4 cc. linseed-ergosterol solution (1) per kilo. . .	20	348	9.9	6.0	43.8
8 cc. linseed-ergosterol solution (1) per kilo. . .	40	370	10.8	6.6	47.7
10 cc. linseed-ergosterol solution (2) per kilo. .	250	401	12.2	7.9	52.7
Ultra violet irradiation 10 min. daily. . . . .	?	395	12.4	8.0	52.7

Curative Trial  
(Chicks on basal ration for 7 weeks before change)

Ration supplements	Vitamin D units fed daily	Days after chg.	Av. final wt.	Blood Analysis		Average ash in tibia
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
None. . . . .	0	0	310	8.7	5.7	42.2
		10	322	9.5	4.2	42.7
		19	351	8.9	4.8	43.0
0.2 cc. linseed-ergosterol solution (1) per chick daily. . . . .	10	10	352	9.9	4.5	42.1
		19	427	10.3	4.9	43.5
0.4 cc. linseed-ergosterol solution (1) per chick daily. . . . .	20	10	347	9.7	4.9	43.3
		19	446	9.5	5.2	45.1
0.4 cc. linseed-ergosterol solution (2) per chick daily*. . . . .	100	10	377	9.6	4.8	44.4
		19	479	11.9	5.3	48.0
Ultra violet irradiation 10 min. daily. . . .	?	10	356	11.3	5.0	44.9
		19	442	11.2	6.6	49.6

\* Solution 2 was five times as concentrated as solution 1.



hand, 20 units daily for a period of 19 days was not sufficient for curing rickets, since a few birds were still affected at the termination of the experiment. All the birds in the lot that received 100 vitamin D units daily, or were subjected to ultraviolet irradiation, had completely recovered by the 19th day. These observations were substantiated by the per cent of bone ash.

Both the prophylactic and curative trials reflected the comparative inefficiency of irradiated ergosterol as an anti-rachitic substance for chicks. In seeking for a possible explanation of the results obtained, it occurred to us that the difficulty, in part, might have been due to the sample of ergosterol or the type of basal ration, which, in our estimation, was too low in phosphorus for practical purposes. Accordingly, another series of several experiments, which involved new samples of irradiated ergosterol and a different rachitic ration, were carried out. The basal ration used throughout was one which gave good growth when properly supplemented with vitamin D. It consisted of ground yellow corn 46, ground wheat 20, wheat bran 5, soybean oilmeal 20, dried buttermilk 5, steamed bone meal 3, and sodium chloride 1. This ration in the different experiments varied between 1.12–1.36 per cent calcium and 0.88–0.98 per cent phosphorus, or a Ca:P ratio of 1.27–1.40.

The chicks in all trials were White Leghorns. They were started on their respective rations when day-old. They were of our own hatching and came from parent stock which had regularly been fed 1 per cent of cod liver oil in their ration. In all instances the chicks were brooded indoors on hardware cloth. Special precautions were taken to exclude all sunlight, by covering the windows with heavy brown wrapping paper. At the start of each trial a few more chicks than were to be carried through the experiment were placed in each lot. All lots were culled to an equal number at the end of seven days, by eliminating those chicks which showed any signs of weakness.

At the end of six weeks, ten or more representative chicks, both males and females, were selected from each lot and killed for blood and bone analysis. The blood from each lot was pooled, refrigerated over night, and the serum analyzed for calcium (6) and inorganic phosphorus (7). Both tibiae from each bird were saved for ash determinations. In removing the adhering flesh, the proximal cartilage was also removed. The bones were then dried, crushed, and wrapped in individual filter papers and extracted in Soxhlet extractors with alcohol and ether. The per cent ash is expressed on a moisture-and fat-free basis.

The first experiment in this series was in part a repetition of the initial work with the inclusion of a cod-liver-oil-fed lot. It consisted of 6 lots of 25 chicks each. A new solution of irradiated ergosterol in linseed oil was used. The ergosterol solutions were diluted so that 1 per cent of the oil solution furnished the respective number of rat units. The results are shown in Table II.

TABLE II  
THE EFFECTIVENESS OF IRRADIATED ERGOSTEROL FOR CALCIFICATION IN THE CHICK

Additions to basal ration	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibia
			Ca. per 100 cc. serum	P. per 100 cc. serum	
		gm.	mg.	mg.	per cent
None (negative control) . . . . .	0	174	7.1	7.1	42.3
Ultra violet irradiation* . . . . .	?	286	9.2	7.7	51.8
2.00 per cent cod liver oil . . . . .	50	288	10.0	7.8	51.1
0.25 per cent ergosterol-linseed oil solution . . .	83	268	9.2	8.1	49.7
0.50 per cent ergosterol-linseed oil solution . . .	166	299	10.8	8.2	50.8
1.00 per cent ergosterol-linseed oil solution . .	332	312	9.3	8.0	51.5

\* Chicks irradiated for 10 minutes three times per week.

The chicks in the negative control lot gave evidence of leg weakness at the fourth week and at six weeks all the birds were severely affected. No external symptoms of leg weakness were observed in any of the other lots except two cases in the lot that received 83 units of vitamin D as irradiated ergosterol in 100 gm. of ration. These observations are substantiated by the bone ash values. The blood analysis of all lots was normal, except the negative control lot, which showed lowered calcium and phosphorus. The results showed that it required between 83 and 166 rat units of vitamin D as irradiated ergosterol per 100 gm. of feed to protect the chick against rickets.

The results of the two trials, in general, were in accord with the findings of other investigators; that it required more equivalent rat units of vitamin D in the form of irradiated ergosterol than cod liver oil. The data, however, did not lend themselves to a direct comparison of the number of units of the vitamin that were required from cod liver oil or the irradiated sterol; since the cod liver oil (2 per cent) apparently had been fed in excess of requirements. Before any fair comparison of the two products could be

made, it was necessary to determine the minimum number of units of either source of vitamin D that were required for normal calcification. It also occurred to us that cod liver oil might carry some factor which would enhance the antirachitic action of the irradiated ergosterol.

To answer these questions 12 lots of 20 chicks each were started on experiment. Three lots were fed the basal ration supplemented with 0.25, 0.50, and 1.00 per cent of cod liver oil. The latter represented a new sample which had a potency of 28 rat units per gram. To the ration of four other lots were added 0.01, 0.05, 0.10, and 0.25 per cent of the same cod liver oil to which had been added irradiated ergosterol to furnish 2, 10, 20, and 50 rat units of vitamin D per 100 gm. of ration, respectively; in addition to the units supplied by the cod liver oil. Four other lots received the basal ration fortified with such amounts of irradiated ergosterol dissolved in corn oil to furnish 1.5, 36, 72, and 143 rat units per 100 gm. of ration, respectively. The remaining lot served as a no-vitamin D control. All three products were evaporated on the basal ration with ether. Fresh feed was prepared every 7-10 days. The results are recorded in Table III.

The chicks in Lots 1, 5, 9, and 10, which received the unsupplemented ration, 2 units of irradiated ergosterol in cod liver oil, and 1.5 and 36 units of activated sterol in corn oil per 100 gm. of ration, respectively, showed marked symptoms of rickets, which were reflected in the low ash content of the tibiae and the lowered calcium values of the blood serum. A few birds in Lots 6 and 11, 0.05 per cent fortified cod liver oil and 0.50 per cent corn oil-sterol solution, respectively, exhibited mild symptoms of vitamin D deficiency. The ash percentage of these lots is also slightly below normal. None of the other lots showed any signs of deficiency and their ash values were normal. It is apparent from these results that it required approximately 7 rat units of vitamin D or less per 100 gm. of ration from cod liver oil and between 72 and 143 rat units of sterol origin to promote normal calcification. On account of the apparent greater efficiency of the natural vitamin D of cod liver oil, it required a smaller number of units as irradiated ergosterol for normal calcification when part of the antirachitic activity was derived from the natural source. The ration of Lot 7, containing 3 units from cod liver oil and 20 units from ergosterol, gave ash values comparable to the ration of Lots 2 and 12, which received 7 units from cod liver oil and 143 units from irradiated sterol in corn oil, respectively.

In order to verify the results of the previous experiment and obtain more definite information on the comparative antirachitic efficiency of irradiated ergosterol dissolved in corn oil and cod liver oil and on the

TABLE III  
THE COMPARATIVE EFFICIENCY OF VITAMIN D FROM COD LIVER OIL AND IRRADIATED  
ERGOSTEROL IN CORN OIL AND COD LIVER OIL

Lot No.	Additions to ration	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None.....	0	271	7.2	6.8	42.7
2	0.25 per cent cod liver oil.....	7	357	11.2	8.7	52.1
3	0.50 per cent cod liver oil.....	14	360	10.9	8.5	51.8
4	1.00 per cent cod liver oil.....	28	375	11.2	8.5	52.0
5	0.01 per cent ergosterol—cod liver oil solution.....	(0.3)+ 2	314	9.1	7.6	45.8
6	0.05 per cent ergosterol—cod liver oil solution.....	(1.5)+10	361	11.4	8.0	49.3
7	0.10 per cent ergosterol—cod liver oil solution.....	(3) +20	360	11.6	8.7	51.4
8	0.25 per cent ergosterol—cod liver oil solution.....	(7)+50	376	11.7	8.8	53.3
9	0.01 per cent ergosterol—corn oil solution.....	1+	229	7.2	5.6	43.1
10	0.25 per cent ergosterol—corn oil solution.....	36	262	8.5	7.3	42.8
11	0.50 per cent ergosterol—corn oil solution.....	72	329	10.7	8.6	49.1
12	1.00 per cent ergosterol—corn oil solution.....	143	343	10.5	8.0	50.3

Note: In Lots 5, 6, 7, and 8 the vitamin D units furnished by the cod liver oil and the irradiated ergosterol are indicated separately. Thus, (3)+20 (Lot 7) implies 3 units from cod liver oil and 20 from the sterol.

minimum number of rat units of the different products that were required for normal calcification in the chick, another experiment was initiated. New samples of cod liver oil and irradiated ergosterol in corn oil were used. Two different strength solutions of irradiated ergosterol in cod liver oil were fed. One represented a 0.01 per cent solution of the sterol and the other a 0.10 per cent solution. Cod liver oil from the same supply as fed unfortified was used in making the solutions. The irradiated ergosterol in corn oil represented a 0.10 per cent solution. The cod liver oil assayed 40 rat units per gram and the irradiated ergosterol 1,666,666 rat units per gram of the sterol. Each lot contained 17 chicks. The solutions were evaporated on fresh portions of ration every 7-10 days.

TABLE IV

THE CALCIFYING VALUE OF VITAMIN D FROM COD LIVER OIL AND IRRADIATED ERGOSTEROL IN CORN OIL AND COD LIVER OIL

Lot No.	Additions to ration, in per cent	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None . . . . .	0	182	5.6	6.2	41.8
2	0.05 cod liver oil . . . . .	2	274	9.6	6.8	46.1
3	0.10 cod liver oil . . . . .	4	313	10.1	6.8	49.4
4	0.20 cod liver oil . . . . .	8	297	11.1	7.5	50.2
5	0.30 cod liver oil . . . . .	12	339	11.4	7.2	51.6
6	0.50 cod liver oil . . . . .	20	310	10.5	8.4	50.1
7	0.03 *fortified cod liver oil . . . . .	(1)+ 50	310	9.9	8.1	49.5
8	0.05 fortified cod liver oil . . . . .	(2)+ 83	310	9.7	8.0	51.3
9	0.10 fortified cod liver oil . . . . .	(4)+166	290	10.3	8.1	50.0
10	0.20 fortified cod liver oil . . . . .	(8)+332	317	9.9	7.3	50.1
11	0.03 †diluted fortified cod liver oil . . . . .	(1)+ 5	289	9.5	7.7	49.7
12	0.05 diluted fortified cod liver oil . . . . .	(2)+ 8	302	10.1	7.6	50.0
13	0.10 diluted fortified cod liver oil . . . . .	(4)+ 16	295	10.1	7.9	51.5
14	0.20 diluted fortified cod liver oil . . . . .	(8)+ 33	314	10.8	7.9	51.6
15	0.03 ‡irradiated ergosterol in corn oil . . . . .	50	282	9.1	7.6	49.6
16	0.05 irradiated ergosterol in corn oil . . . . .	83	291	10.5	7.5	50.3
17	0.10 irradiated ergosterol in corn oil . . . . .	166	289	10.5	7.7	51.1
18	0.25 irradiated ergosterol in corn oil . . . . .	416	301	10.9	7.7	50.3
19	0.50 irradiated ergosterol in corn oil . . . . .	833	314	10.9	8.0	50.8

\* A 0.10 per cent solution of irradiated ergosterol in cod liver oil.

† A 0.01 per cent solution of irradiated ergosterol in cod liver oil.

‡ A 0.10 per cent solution of irradiated ergosterol in corn oil.

(1)+50=1 unit from cod liver oil and 50 from irradiated ergosterol.

The results summarized in Table IV are self explanatory. All of the chicks that received 0.05 per cent or 2 rat units of vitamin D per 100 gm. of ration from cod liver oil (Lot 2) showed evidence of an antirachitic deficiency; whereas only 3 birds on the 4-unit level (Lot 3) exhibited symptoms of leg weakness. When the amount of cod liver oil was increased to 0.20 per cent, or 8 units, complete protection resulted, as attested by the condition of the birds and the ash content of their tibiae. These findings are in accord with the results of the previous experiment, where 7 rat units of vitamin D per 100 gm. of ration of a different cod liver oil were sufficient for normal calcification. In contrast, it required 83 or more units in the form of irradiated ergosterol in corn oil to produce the same effect. A few

birds on the 50 rat unit ergosterol level (Lot 15) showed visible symptoms of rickets at the sixth week; whereas no evidence of leg weakness and a normal bone ash were observed when 83 or more units of vitamin D were included in the ration. No detrimental or ill effects were noted when an excess or ten times (833 units) the probable minimum requirement of vitamin D were fed as irradiated ergosterol (Lot 19). The feeding of irradiated ergosterol in cod liver oil, either in a 0.01 or 0.10 per cent solution, apparently did not enhance the antirachitic activity of the sterol, because it required considerably more total units of vitamin D in this combination to afford complete protection against rickets than in the form of the untreated cod liver oil. In case of the 0.01 per cent solution of the sterol in cod liver oil it required somewhat more than 2 units from cod liver oil and 8 from activated ergosterol (Lot 12) to give complete protection against rickets. Although the ash content and the blood analysis of this lot appears normal, it was noted that two birds were bordering on leg weakness at 6 weeks. Of the 0.1 per cent ergosterol-cod liver oil solution it required more than 1 unit from the cod liver oil and 50 from the sterol (Lot 7) for maximum calcification.

The results of the foregoing experiment with respect to the comparative effectiveness of irradiated ergosterol in cod liver oil and corn oil, are rather difficult to interpret on account of the increased number of rat units from cod liver oil, irrespective of their greater efficiency, that were fed. To overcome this difficulty cod liver oil from the same supply as used in the former experiment was fortified with such quantities of previously standardized ergosterol that the resulting solution when fed at 0.03 per cent of the ration furnished 5, 10, 25, and 50 rat units of vitamin D per 100 gm. of feed in the form of the sterol in addition to the 1 unit from the cod liver oil. Additional lots on the untreated cod liver oil and the irradiated ergosterol in corn oil were included in the trial.

The results of the blood and bone analysis (Table V) show that it required between 4 and 8 units of vitamin D per 100 gm. of basal ration from cod liver oil to give complete protection; substantiating the results of former trials. Apparently 4 units were very near the minimum requirement, since only two birds showed slight evidence of a deficiency. On the contrary, it required between 75 and 100 rat units of irradiated sterol in corn oil to meet the requirements of the chick. The feeding of irradiated ergosterol in cod liver oil apparently did not influence the antirachitic value of the sterol, since it required 50 or more rat units in the form of ergosterol in addition to the 1 unit supplied by the cod liver oil for normal

TABLE V  
THE ANTIRACHITIC EFFICIENCY OF IRRADIATED ERGOSTEROL AND COD LIVER OIL  
FOR THE CHICKEN

Lot No.	Additions to ration, in per cent	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None . . . . .	0	168	5.8	5.9	39.2
2	0.05 cod liver oil . . . . .	2	261	8.5	5.8	43.3
3	0.10 cod liver oil . . . . .	4	255	10.3	6.4	49.8
4	0.20 cod liver oil . . . . .	8	287	12.2	6.4	50.8
5	*0.015 ergosterol in corn oil . . . . .	25	202	8.4	6.2	43.2
6	0.030 ergosterol in corn oil . . . . .	50	243	11.0	5.8	48.5
7	0.045 ergosterol in corn oil . . . . .	75	261	10.6	6.5	49.9
8	0.060 ergosterol in corn oil . . . . .	100	290	11.1	7.1	50.1
9	0.030 ergosterol in cod liver oil . . . . .	(1)+5	210	8.2	6.4	41.1
10	0.030 ergosterol in cod liver oil . . . . .	(1)+10	231	8.5	6.5	42.0
11	0.030 ergosterol in cod liver oil . . . . .	(1)+25	235	9.7	7.4	47.3
12	0.030 ergosterol in cod liver oil . . . . .	(1)+50	260	10.2	7.1	49.2

\* Represented a 0.1 per cent solution of irradiated ergosterol in corn oil.

The cod liver oil solution in Lots 9, 10, 11, and 12 represented 0.01, 0.02, 0.05, and 0.10 per cent solutions of irradiated ergosterol in the oil, respectively.

(1)+50 = 1 rat unit from cod liver oil and 50 from irradiated ergosterol

calcification. Although there was no evidence of leg weakness in the latter group (Lot 12) the ash values are questionably normal. The results, in general, corroborate the data of the previous experiments.

With the marked difference in the antirachitic value of vitamin D from cod liver oil and irradiated ergosterol, the relative efficiency of irradiated yeast as a source of this factor presented itself. It was also thought advisable to compare the effectiveness of ergosterol prepared from yeast with that prepared from fungus. Accordingly, we proceeded to determine the comparative effectiveness of vitamin D from cod liver oil, irradiated yeast,<sup>1</sup> and irradiated ergosterol of yeast<sup>1</sup> and fungus origin. The products, as in previous experiments, were assayed for anti-rachitic activity on rats prior to the start of the experiment.

The trial included 12 lots of 15 chicks each. One lot was put on the basal ration, two on cod liver oil, three on irradiated yeast, and three on each

<sup>1</sup> We are indebted to Standard Brands, Inc., of New York City, for the irradiated yeast and ergosterol of yeast origin.

sample of irradiated ergosterol in corn oil. To equalize the oil and yeast intake of all lots, 1 per cent of corn oil and 1 per cent of untreated yeast were included in the basal ration; the cod liver oil and irradiated products replacing an equivalent amount of the corn oil or untreated yeast in the basal mixture.

It is evident from the data in Table VI that it required somewhat more than 100 rat units of vitamin D per 100 gm. of feed of either irradiated ergosterol sample for normal calcification. No outstanding difference in the protection afforded by the two different solutions of the irradiated sterol is apparent. Irradiated yeast did not prove so efficient as irradiated ergosterol when fed on an equivalent rat unit basis. This observation is not in accord with the findings of Steenbock, Kletzien, and Halpin (5) who reported that it required from 7.5 to 60 per cent cod liver oil equivalence as irradiated yeast and from 40 to 120 per cent cod liver oil equivalence as irradiated ergosterol for normal bone production. The superiority of the natural vitamin D from cod liver oil is again demonstrated—requiring approximately 8 rat units or less per 100 gm. of ration for normal protection. The blood analyses, in general, approximate the ash determinations.

It should be noted that the lower per cent of tibia ash in this experiment

TABLE VI

THE EFFECTIVENESS OF VITAMIN D FROM COD LIVER OIL AND IRRADIATED ERGOSTEROL AND YEAST FOR CALCIFICATION IN THE CHICKEN

Lot No.	Substitutions in basal ration, in per cent	Vitamin D rat units per 100 gm. ration	Average final weight	Blood analysis		Average ash in tibiae
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	mg.	mg.	per cent
1	None . . . . .	0	186	7.8	5.1	37.4
2	0.10 cod liver oil . . . . .	4	404	9.6	6.2	43.3
3	0.20 cod liver oil . . . . .	8	473	10.5	7.9	46.9
4	0.02 ergosterol (No. 1) in corn oil . . . . .	50	272	9.2	6.6	39.8
5	0.04 ergosterol (No. 1) in corn oil . . . . .	100	332	10.3	6.2	42.0
6	0.08 ergosterol (No. 1) in corn oil . . . . .	200	390	10.8	7.0	46.9
7	0.015 ergosterol (No. 2) in corn oil . . . . .	50	320	9.8	6.4	42.9
8	0.03 ergosterol (No. 2) in corn oil . . . . .	100	331	11.0	6.4	44.5
9	0.06 ergosterol (No. 2) in corn oil . . . . .	200	393	11.0	6.3	46.1
10	0.15 irradiated yeast, 25 D. . . . .	50	188	8.5	5.2	38.7
11	0.30 irradiated yeast, 25 D. . . . .	100	199	9.6	5.2	39.7
12	0.60 irradiated yeast, 25 D. . . . .	200	313	9.2	6.0	43.8

No. 1 irradiated ergosterol was prepared from yeast, while No. 2 was of fungus origin.



was due to the fact that both cartilages were left intact when the bones were cleaned of adhering flesh, while in the previous experiments the proximal cartilages were removed. We have no explanation for the significantly greater growth obtained in the cod-liver-oil-fed lots. Previous experiments did not show this difference.

### DISCUSSION

The data conclusively show that irradiated ergosterol and irradiated yeast are considerably less effective than the rat equivalent amount of cod liver oil for calcification in the chicken. In this regard, the data substantiate the findings of other investigators. However, the results of the present investigation show the irradiated products somewhat more efficient than reported by Mussehl and Ackerson (1), Massengale and Mussmeier (2), and Steenbock, Kletzien, and Halpin (5). The reports of these investigators are rather difficult to interpret in terms of our results, in that they did not make comparisons on a minimum protective basis. In general, they indicate that it required from 40 to 100 times as many rat units of the irradiated products as from cod liver oil, whereas our results only show an approximate 15 to 20 fold increase.

It is interesting to speculate why the chicken is not able to utilize vitamin D from the three sources equally as efficiently as the rat. If it is a question of species difference, are the results due to a difference in amount or degree of absorption of the vitamin, or to an actual difference in the vitamin D substance? Klein and Russell (8) have reported that they were able to recover 26.5 and 43.1 per cent of the total vitamin D units fed to chicks as irradiated ergosterol in corn oil and cod liver oil, respectively. This would suggest that the difference in antirachitic effectiveness is not one of absorption. On the other hand, if the difference in efficiency is one of substance, we must assume, like Steenbock, Kletzien, and Halpin (5), that the natural vitamin D from cod liver oil and irradiated yeast and ergosterol are not identical. Work by English (9) and German (10) investigators shows that there are two forms of vitamin D in ordinary irradiated ergosterol. Accordingly, there is the possibility that one form might prove more efficient than the other in case of the chicken while both might serve equally efficiently for the rat.

Considerable study has been devoted to the effect of vitamin A on the comparative efficiency of cod liver oil and irradiated ergosterol. Poulsson (11) suggested that the vitamin A of cod liver oil was responsible for the superior effect of cod liver oil over ergosterol. Russell and Klein (4) and

Steenbock, Kletzien, and Halpin (5), respectively, on the contrary, found that dried yellow carrots or carotene failed to increase the effectiveness of the irradiated sterol for chicks. Likewise, Hunter, Dutcher, and Knandel (12), who used alfalfa meal as an additional source of vitamin A, report negative results. Apparently vitamin A is not a factor in the comparative antirachitic efficiency of irradiated ergosterol for the chicken.

#### SUMMARY

1. Irradiated yeast and irradiated ergosterol dissolved in linseed oil, corn oil, or cod liver oil are less effective than the rat equivalent amount of cod liver oil for calcification in the chicken.

2. It required from 15 to 20 times as many rat units of vitamin D per unit of feed in the form of irradiated ergosterol as in the form of cod liver oil for normal bone formation.

3. Cod liver oil did not increase the antirachitic efficiency of irradiated ergosterol for the chicken.

4. Irradiated ergosterol proved somewhat more efficient than irradiated yeast when equivalent rat units of vitamin D were fed.

5. Under the conditions of the experiments, the chick required a minimum of approximately 7 rat units of vitamin D per 100 gm. of ration from cod liver oil for normal calcification.

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# LATHYRISM IN THE RAT

By

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LATHYRISM, which has been known since the time of Hippocrates, has been very common in the past in India and northern Africa, and small local outbreaks have occurred frequently in Italy, France, and elsewhere in southern Europe (1). For a long time it has been known that the disease is caused by the eating of lathyrus peas of three species, *Lathyrus sativus*, *Lathyrus cicera*, and *Lathyrus clymenum*. After a poor crop of wheat, barley, and other cereals, the poorer people in India and some parts of northern Africa are forced to eat the lathyrus peas as a large part of their diet, and lathyrism then becomes prevalent. The use of the peas as one-third to one-half of the diet for two or three months is considered enough to cause the disease (2). However, not all persons eating such a diet are affected, and in families only certain members are attacked. When the peas are eaten as a smaller proportion of the diet, they are said to be harmless (1).

The outstanding symptom of lathyrism in man is a spasticity and rigidity of the leg muscles which results in a jerky gait with flexed knees, or an inability to walk. The condition develops very rapidly and is often precipitated by exposure to cold, wet, and fatigue. Death seldom results, but there is usually a permanent lameness (1). The symptoms have suggested to various workers the possibility that they may be due to degenerative changes in the spinal cord. In fact Mellanby (3) has gone so far as to suggest that vitamin A may be able to prevent this degeneration. However, he has not reported any experiments to test this idea so far as we are aware.

Different animals are said to vary greatly in their susceptibility to the poisonous action of the lathyrus peas. In Great Britain peas of this type are used as food for farm animals. When mixed in the ration in not too

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large proportion they are said to give satisfactory results, although occasionally bad effects have been reported (1). Of the farm animals, horses have been found to be peculiarly susceptible. Stockman (1) stated that 20 per cent of lathyrus peas in the diet is harmful to horses, while pigs and cattle can tolerate higher levels. Feeding experiments have been carried out using monkeys, rabbits, guinea pigs, rats, pigeons, and ducks. According to Stockman (4) monkeys have spasmodic attacks of a dramatic kind occurring at irregular intervals, in addition to the paralysis. He reported that death was due to failure of the respiratory muscles. In his experiments on rabbits, in which he used seven animals, he observed in some cases a very transient and slight spasmodic condition of the legs, but most of his animals grew normally. He assumed the presence of a toxin and inferred that rabbits are susceptible to the toxin, but that in ordinary feeding experiments they get too little poison to produce symptoms. In four out of five guinea pigs he noted tremors, spasticity, and weakness of the legs and reported that all died in 8 to 35 days. He stated that "the lethal action is not a specific lathyrus action, but seems to be due to the fact that the proteins of lathyrus peas cannot be assimilated and utilized by guinea pigs." McCarrison (5) reported that he was unable to cause lathyrism in rats even when feeding lathyrus peas as the only food. Zagami (6) also failed to secure symptoms characteristic of lathyrism in rats. He fed a diet made up entirely of lathyrus peas for 150 days. He did report a decrease in the rate of gain, retardation in sexual development, and a decrease in calcification of the skeleton, which can be readily understood, inasmuch as all seeds when fed by themselves are seriously deficient in various nutritive elements. Acton is said to have produced paralysis in ducks by feeding them lathyrus peas (1). Pigeons have been reported insusceptible to the toxin (4).

The discrepancies in the results of different investigators are difficult of interpretation. Undoubtedly many of them were due to the use of poor basal diets which frequently were deficient in mineral elements, vitamin D, and good protein, and probably were otherwise poorly constituted. Mellanby (3), for instance, believes that vitamin A furnished variable protection depending on the amount present. Emphasis should, however, also be placed on the fact that the investigators frequently used different species and varieties of lathyrus peas and no mention was made of the conditions under which they were grown.

There is a difference of opinion among experimenters as to the nature of the toxic substance in the lathyrus peas. Stockman (7) asserted that

the toxic principle was an alkaloid which he obtained in very small amount from the peas and with which he could produce paralysis in frogs, monkeys, and mice. However, Acton and Chopra insisted that an amine and not an alkaloid was the toxic substance (cited from [1]). These investigators reported paralysis in the hind legs of monkeys, guinea pigs, rats, and mice with the pure hydrochloride of the amine obtained from the peas. In contrast to both of these views, Anderson, Howard, and Simonsen (8) are of the opinion that the poisonous effect was due to other seeds, especially *Vicia sativa* variety *Augustifolia*, found mixed with the lathyrus peas. They observed no toxic effects of any kind when botanically pure *Lathyrus sativus* seeds were fed to animals, but stated that when seeds of *Vicia sativa* were fed to monkeys, nervous and muscular symptoms resulted, and when fed to ducks they caused death. *Vicia sativa* was reported as containing bases showing alkaloidal properties of which they isolated three, vicine, divicine, and a cyanogenetic glucoside, vicianin, at least one of which, divicine, produced characteristic symptoms when injected into guinea pigs. A still different theory of the cause of lathyrism was advanced by Young (2) after he made a field study of the disease in India. He suggested that the disease was due primarily to a deficiency of vitamin A.

From the preceding it is evident not only that there is considerable difference of opinion regarding the etiology of lathyrism, but also that the properties of different species are very different and hardly specific.

In the experiments detailed in the following report the effect of feeding lathyrus peas to rats was determined. An attempt was made to ascertain whether the abnormal condition produced by the peas was ameliorated or prevented by the feeding of cod liver oil or yeast as vitamin supplements. The species of lathyrus peas which was used in our experiments was *Lathyrus odoratus*, the sweet pea used for ornamental purposes in this country.

#### EXPERIMENTAL

The composition of the diets is shown in Table I.<sup>1</sup> The casein was a commercial product freed from vitamin A by heating it at 90°C. for two weeks. The dextrin in reality represented cooked starch. It was prepared by mixing enough water with cornstarch to make lumps, autoclaving it for 2

<sup>1</sup> We wish to thank John Bodger and Sons of El Monte, California, for the sweet peas, E. R. Squibb and Sons for the cod liver oil, and National Oil Products Company for the cod liver oil concentrate used in these experiments.

TABLE I  
COMPOSITION OF DIETS\*

Lots	1	3	27	28†	29†	51	52	55	57	77	79	81	82	83	117‡	121§
Sweet peas	—	50	50	50	50	25	25	50	50	50	—	12.5	5	50	—	50
Casein (Vitamin A free)	10	10	10	10	10	10	10	10	10	—	18	14	14	—	18	10
Salts 40	4	4	4	4	4	4	4	4	4	—	4	4	4	—	4	4
Dextrin	34	36	34	36	34	61	59	33.9	26	—	68	63.5	71	—	71	35
Cod liver oil	2	—	2	—	2	—	2	2	—	—	2	—	—	2	1	1
Cod liver oil concentrate	—	—	—	—	—	—	—	0.1	—	—	—	—	—	—	—	—
Yeast	—	—	—	—	—	—	—	—	10	—	8	6	6	—	6	—
Garden peas	50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Yellow corn	—	—	—	—	—	—	—	—	—	26	—	—	—	24	—	—
Wheat gluten	—	—	—	—	—	—	—	—	—	20	—	—	—	20	—	—
CaCO <sub>3</sub>	—	—	—	—	—	—	—	—	—	3	—	—	—	3	—	—
NaCl	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—	—

\* All rations except 78 contained 50 mgs. irradiated yeast per 100 gms. of ration.

† Rations 28 and 29 were made up with peas which had been cooked for 2½ hours.

‡ Ration 117 had the water extract from 100 gms. of sweet peas evaporated on 71 parts of dextrin.

§ Ration 121 contained sweet peas which had been boiled and thoroughly extracted with hot water.

|| Steenbock, H., and Nelson, E. M., *Jour. Biol. Chem.*, 1923, 66, 355.

hours at 15 lbs. pressure, drying it, and then grinding. The cod liver oil concentrate was incorporated in the ration by dissolving it in ether, pouring the ether solution on part of the dextrin and then evaporating off the solvent in a current of air. This was then mixed with the other ingredients of the ration. The yeast was obtained from the Northwestern Yeast Company and had been previously tested in this laboratory for its vitamin B content. The irradiated yeast was yeast which had been irradiated for 2 hours with an Alpine Sun Lamp at a distance of 18 inches with stirring.

The rations were so outlined as to be adequate in all respects, except that in some, vitamin A was absent aside from such amounts as were contained in the sweet peas themselves. For control purposes to demonstrate the general adequacy of the supplements, garden peas were substituted for the sweet peas at the higher levels and 2 per cent of cod liver oil was included. With these in the diet approximately normal growth was obtained and the animals maintained themselves in excellent condition for 14 weeks. At this time the trials were terminated. The rations were fed *ad libitum*, the food consumption being recorded during the first 4 or 5 weeks of the experiments. Male albino and piebald rats weighing between 50 and 60 grams when 3 to 4 weeks old were used in lots of 4. Each rat was kept in a separate cage with a raised screen bottom. Distilled water was given in addition to the ration. The animals were weighed once a week.

Contrary to the findings of McCarrison (5) and Zagami (6) we have succeeded in inducing lathyrism in rats. Very little growth occurred on the diets which contained 80 per cent of sweet peas and the rats lived for only 4 to 6 weeks (Lot 4). As the percentage of sweet peas in the diet was decreased, greater growth resulted. Of the 40 animals receiving 50 per cent of sweet peas from the time of weaning, 3 grew to a weight of 160 grams, during 3-1/2 months on the ration; the rest weighed less than this. One of the 8 animals on the 25 per cent level attained a weight of 215 grams. However, when the sweet peas were fed as 12.5 per cent of the ration or less, practically normal growth was secured for 20 weeks in a group of 8 rats. The growth of the animals is summarized in Table II.

Depression of growth was only one effect of the diets containing sweet peas. The first sign of abnormality appeared after about 4 weeks on diets containing 50 per cent of sweet peas and after 6 weeks on those containing 25 per cent. All the animals developed a marked spinal curvature. The long bones were variously deformed, and the rat frequently walked with a shambling gait. The abnormalities became progressively worse and apparently the animals were in discomfort, as they became very inactive.



TABLE II  
GROWTH OF RATS ON SWEET PEA DIETS  
(Average weights)

Lot No.	Character of ration	Weight					Remarks
		Initial	at 4 weeks	at 6 weeks	at 10 weeks	at 15 weeks	
1	50% garden peas+2% C.L.O.	gms. 56	gms. 113	gms. 136	gms. 168	gms.	Control group. Approximately normal growth. In excellent condition when etherized after 13 weeks.
4	80% sweet peas	52	75				Very little growth. Digestive disturbance (flatulence, diarrhea). Respiratory difficulty after 3 weeks. Hind legs of 2 rats paralyzed after 5 weeks. All died during 5th or 6th week.
3	50% sweet peas	54	104	112	123		Slow growth for 4 to 6 weeks. Hind legs lame after 4 weeks, those of 2 rats in Lot 3 almost completely paralyzed. Lordosis in thoracic region. Sternum misshapen in 2 rats. Some digestive disturbance. 3 died between 7th and 12th weeks, others etherized.
26	50% sweet peas	55	111	123	118		
27	50% sweet peas +2% C.L.O.	55	103	125	143		Some improvement in growth with addition of C.L.O. Lameness and lordosis after 4 or 5 weeks. Hind legs partially or completely paralyzed. All rats in Lot 27 developed hernias. Ventral or lateral spinal curvature in all. 4 died between 5th and 12th weeks, others etherized.
39	50% sweet peas +2% C.L.O.	55	85	114	143		
50	50% sweet peas +2% C.L.O.	58	107	132	143		
55	50% sweet peas +2% C.L.O.+ 0.1% C.O.C.†	54	101	113			No better growth than Lots 27, 39 and 50. Lameness and lordosis after 5 weeks. One rat developed a hernia. Etherized after 7 weeks.
57	50% sweet peas +10% yeast	57	121	132	127		No better growth than Lots 3 and 26. Symptoms of lathyrism after 5 weeks. All died between 6th and 15th weeks.

† C.O.C. = Cod liver concentrate.

TABLE II (Continued)

Lot No.	Character of ration	Weight					Remarks
		Initial	at 4 weeks	at 6 weeks	at 10 weeks	at 15 weeks	
		gms.	gms.	gms.	gms.	gms.	
51	25% sweet peas	54	91	118	158	168	Better growth than with 50%. Growth fairly good for 12 weeks with no C.L.O. in diet. Symptoms of lathyrism after 6 weeks. Etherized after 18 weeks.
52	25% sweet peas +2% C.L.O.	54	89	112	159	176	Slightly better growth than Lot 51. Symptoms of lathyrism in 3 rats after 6 weeks, in one after 9 weeks. Etherized after 18 weeks.
81	12.5% sweet peas	53	139	185	249	277	Normal growth with no source of vitamin A except sweet peas. No symptoms of lathyrism after 20 weeks. Yeast included after 5 weeks to supplement vitamin B in sweet peas since appetite was declining. Etherized after 20 weeks. Slight enlargement of costochondral junctions on autopsy.
82	5% sweet peas	57	117	182	208	242	
28	50% sweet peas (cooked)	52	92	100	100		Same results as with uncooked sweet peas. 5 died between 5th and 12th weeks, others etherized after 12 weeks.
29	50% sweet peas (cooked) +2% C.L.O.	55	102	117	135		
73	50% sweet peas	359	345	348	347	350	Weight maintained. Lameness appeared in all rats. Etherized after 15 weeks.
74	50% sweet peas +2% C.L.O.	343	340	352	351	346	Slight increase in weight. No lameness developed. Etherized after 15 weeks.
75	25% sweet peas	383	363	372	366	377	Weight maintained. No symptoms of lathyrism. Etherized after 15 weeks.
76	25% sweet peas +2% C.L.O.	342	337	346	347	349	Slight increase in weight. No symptoms of lathyrism. Etherized after 15 weeks.

TABLE II (Continued)

Lot No.	Character of ration	Weight					Remarks
		Initial	at 4 weeks	at 7 weeks	at 10 weeks	at 15 weeks	
78	50% sweet peas (no vitamin D.)	51	90	111	126		Slightly poorer growth than Lots 3 and 26 which received irradiated yeast. All died between 3rd and 14th weeks.
77	#2965+50% sweet peas	54	91	88	79		Lathyrism after 3 weeks. Six died between 6th and 14th weeks, others etherized.
83	#2965+50% sweet peas+2% C.L.O.	55	82	93	102		
79	Control Diet	53	146	207	256		Normal growth. Etherized after 11 weeks.
117	Water extract of sweet peas	54	135	129	134		Good growth for 4 weeks, then decline. Lathyrism after 4 weeks. All died between 7th and 12th weeks.
121	50% water extracted sweet peas	54	73	116	203		Good growth. No symptoms of lathyrism after 11 weeks. Experiment continuing.

In a few cases the hind legs were completely paralyzed. In 2 cases the front legs were also involved, the feet turning out at the wrists. In many of the animals the sternum was deformed. For a typical illustration of such animals see Plate 1.

Although hernia in the rat is a very unusual phenomenon, practically never occurring in the stock colony and to the best of our knowledge never having been reported in relation to dietary abnormality in the rat, it occurred in 10 cases, or 25 per cent of the young animals receiving the 50 per cent level of sweet peas. Some of the hernias were permanent while others appeared and disappeared at intervals. The contents of the hernias varied, sometimes consisting of the cecum and part of the intestines only, while in others a kidney, a testis, and even the stomach and spleen protruded in addition.

The severity of the skeletal changes became evident on autopsy. The spinal column in every case showed extreme curvature in the thoracic

region. In most of the animals the curvature was only ventral although it sometimes was also lateral. Invariably the ventral curvature involved



PLATE 1.—A side view of one rat from Lot 3 showing the spinal curvature characteristic of animals on diets containing 50 and 25 per cent of sweet peas.

the lower seven thoracic vertebrae. An outstanding feature was the short radius which the curvature assumed even in the larger animals. This did

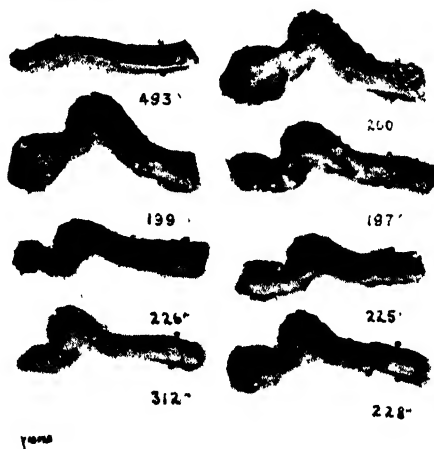


PLATE 2.—Photographs of vertebral columns showing curvatures in the thoracic region. These photographs were taken with the ventral side uppermost and the cervical end to the left. Rat 493—a normal animal from the stock colony. Rats 197, 199, 200 from Lot 30 received a diet containing 50 per cent of sweet peas and 2% of cod liver oil. Rats 225, 226, 228 from Lot 57 received a diet containing 50 per cent of sweet peas and 10 per cent of yeast. Rat 312 from Lot 78 received 50 per cent of sweet peas.

not exceed  $3/8$  of an inch (Plate 2). In most cases there was also a dorsal curvature in the lumbar region. In some rats the tail made an acute angle with the body.

Although the spinal curvature dominated the picture the thoracic cavity was otherwise affected. In the first place the sternum was frequently deformed. Sometimes it assumed a dorsal-ventral curvature, but in other cases it was also deformed laterally. It was noted that in addition the ribs were frequently misshapen. Sometimes the costochondral junc-

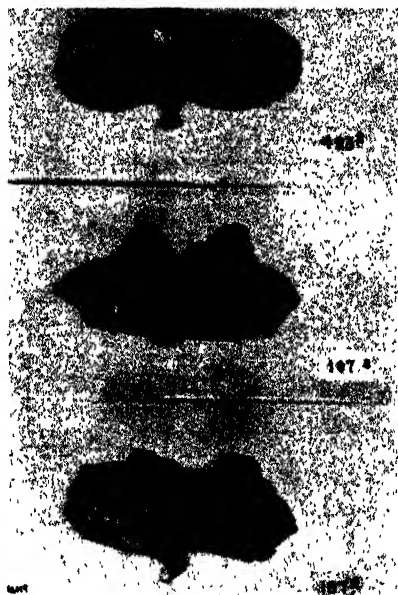


PLATE 3.—Photographs showing malformation of ribs and sternums. Rat 493—A normal animal from the stock colony. Rats 107 and 197—From Lots 27 and 50 respectively received a diet containing 50 per cent of sweet peas and 2 per cent of cod liver oil. Note angulation at the costochondral junctions and deformation of the sternum.

tions were merely enlarged, but in other cases angulation was also pronounced. The photographs shown in Plate 3 may be taken as typical examples. It was evident that these changes could not have been caused by rickets, since all the rations contained 4 per cent of a complete salt mixture as well as vitamin D in an amount previously shown in this laboratory to be ample for protection.

The femora and humeri were badly deformed. The upper ends of the femora were broadened and rough areas were present on either side at the points of attachment of the muscles. The humeri were also thickened and

showed similar roughened areas (Plate 4). Taking an average of 30 animals and comparing them with a similar number which had similar weights recorded by Donaldson (9), the weights of femora and humeri were found to be 33 and 26 per cent less, respectively.

In view of the deformities both in the vertebral column and in the long bones it was believed possible that changes in mineralization of the bones had occurred. However, that this was not extreme was evident from the fact that the bones were observed to be neither brittle nor flexible. Deter-

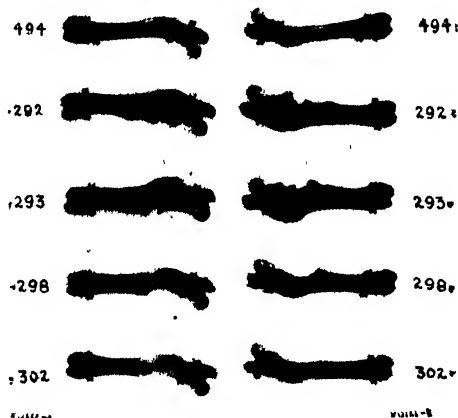


PLATE 4. Photographs of femora from adult rats which had received diets containing 25 and 50 per cent of sweet peas for 16 weeks after they had attained a weight of 300 to 400 grams. The femora at the left show the appearance of the dorsal surface of the bones while those on the right show the ventral surfaces.

Rat 494—A control animal from the stock colony. Rat 292 from Lot 73 received 50 per cent of sweet peas. Rat 293 from Lot 74 received 50 per cent of sweet peas and 2 per cent of cod liver oil. Rat 298 from Lot 75 received 25 per cent of sweet peas. Rat 302 from Lot 76 received 25 per cent of sweet peas and 2 per cent of cod liver oil.

Note the abnormal shape of the bones and the exostoses at the points of attachment of the muscles. The bones of the animals which received 25 per cent of sweet peas are less abnormal than those on the 50 per cent level. The addition of cod liver oil to the rations prevented the bone changes to some extent.

minations of ash were made in the usual manner. The bones were dissected out immediately after death, extracted with alcohol and ether, dried, and ashed. The results are presented in Table III. It will be noted from the

table that the ash content of the bones of the adults falls within the normal limits. In the young both the amount of ash and percentage of ash were decreased.

TABLE III  
PERCENTAGE OF ASH IN THE BONES OF RATS ON SWEET PEA DIETS

Lot	No. of femora	Sweet pea additions	Average age	Average weight	Weight of ether extracted femur	Weight of ash	Ash
			days	gms.	gms.	gms.	%
26, 28	3	50%	113	116	0.317	0.168	52.8
27, 29, 39	6	50%+2% C.L.O.	100	136	0.337	0.177	52.8
55	2	50%+2% C.L.O.+ 0.1% C.O.C.†	77	116	0.287	0.149	52.0
57	1	50%+10% yeast	114	107	0.299	0.173	58.0
51	1	25%	161	198	0.530	0.336	63.3
52	3	25%+2% C.L.O.	156	209	0.471	0.293	62.1
73	2	50%	274	354	0.651	0.400	61.8
74	2	50%+2% C.L.O.	281	336	0.687	0.420	61.0
75	2	25%	247	355	0.631	0.394	62.5
76	2	25%+2% C.L.O.	257	340	0.635	0.416	65.5
Stock	15	Stock ration only	101	294	0.455	0.283	62.2
Stock	14	Stock ration only	201	360	0.544	0.338	61.9

† C.O.C. = Cod liver oil concentrate.

Inasmuch as the blood phosphorus and calcium relations have served as valuable indices of the state of bone metabolism, such data on our animals were obtained. The Fiske-Subbarow method (10) was used for determination of inorganic phosphorus. The serum calcium was determined on the protein-free filtrate obtained after treating the serum with four times its volume of 10 per cent trichloroacetic acid. Aliquots corresponding to 1 cc. of serum were used. The hydrogen ion concentration was adjusted in the manner used by McCrudden (11) for organic materials. The calcium was precipitated with ammonium oxalate and after treatment with normal  $\text{H}_2\text{SO}_4$  was titrated with  $\text{N}/100 \text{ KMnO}_4$ , using a micro-burette. The values obtained are tabulated in Table IV. Inspection of the collected data on the adults reveals that the serum calcium and inorganic phosphorus were within the normal limits. Unfortunately blood analyses on young rats were not made.

One of the outstanding changes in the bones was their congested appearance. Distinct hemorrhages were also noted macroscopically in the

muscle and connective tissue immediately contiguous to the roughened areas on some of the femora and humeri. The appearance of the bones themselves suggested a possible stimulation of hematopoietic activity. Individual determinations of hemoglobin by the Newcomer method, using blood obtained from the tails of 23 of the animals, gave results ranging from 12.2 to 15.9. Three-fourths of the values fell above 14, which in-

TABLE IV  
MILLIGRAMS PER CENT OF CALCIUM AND PHOSPHORUS IN THE BLOOD OF RATS  
ON SWEET PEA DIETS

Lot	No. of rats	Sweet pea additions	Serum Ca	Serum P
			mg. %	mg. %
73	4	50%	10.4	6.9
74	4	50%+2% C.L.O.	11.2	7.0
51	4	25%	10.0	9.1
75	3	25%	9.9	6.9
76	4	25%+2% C.L.O.	10.0	6.6
52	4	25%+2% C.L.O.	10.2	9.6

dicates a tendency toward a high level. The erythrocyte count of these same animals estimated in a Levy counting chamber with single Neubauer ruling averaged 9,900,000 with a range of 7,800,000 to 12,000,000. All but 3 counts were above 9 million and 10 were above 10 million, thus falling in the upper part of the normal zone or slightly above. Averaged values of three to four animals in each group are shown in Table V. Examination of the spleens showed them to be somewhat smaller in pro-

TABLE V  
HEMOGLOBIN VALUES AND ERYTHROCYTE COUNTS OF RATS ON SWEET  
PEA DIETS

Lot	No. of rats	Sweet pea additions	Hemoglobin, gms. per 100 cc. of blood	Erythrocytes per cu. mm. of blood
73	4	50%	15.48	9,863,000
74	4	50%+2% C.L.O.	14.75	10,163,000
51	4	25%	14.20	8,850,000
75	3	25%	14.02	10,850,000
76	4	25%+2% C.L.O.	13.55	10,330,000
52	4	25%+2% C.L.O.	14.26	9,300,000



portion to the body weight, especially in those animals receiving the 50 per cent level of sweet peas (Table VI).

TABLE VI  
THE WEIGHT OF THE SPLEEN OF RATS ON SWEET PEA DIETS

Lot	No. of rats	Sweet pea additions	Range of body weight	Average spleen weight	Average body weight divided by average spleen weight
			gms.	gms.	
73	4	50%	294-400	0.740	475
26	2	50%	125-147	0.270	507
27	1	50%+2% C.L.O.	147	0.311	471
29	1	50%+2% C.L.O.	140	0.367	381
50	4	50%+2% C.L.O.	123-151	0.251	549
55	4	50%+2% C.L.O.+0.1% C.O.C.†	109-120	0.589	243
57	3	50%+10% yeast	99-138	0.372	343
75	3	25%	351-427	0.624	607
51	4	25%	130-198	0.461	375
76	4	25%+2% C.L.O.	313-384	0.731	458
52	4	25%+2% C.L.O.	163-215	0.683	233
79	3	Control diet	186-400	0.683	398

† C.O.C. = Cod liver oil concentrate.

To supplement our observations on young animals a few experiments were carried out on old rats. They were put on the ration at a weight of 300 to 400 grams. Some of the gross effects obtained with the young animals were secured with such adults. The femora and humeri became markedly congested, the muscular attachments were hemorrhagic and the cortex was roughened. All the long bones were deformed. Lameness became incident after 12 weeks in the animals which received 50 per cent of sweet peas with no vitamin supplement. The essential differences were that the spinal columns, the ribs, and sternums were normal. Hernia also did not develop.

To pave the way for further studies on lathyrism, but not in direct relation to our immediate problem, an attempt was made to inactivate the toxic principle in the sweet peas by cooking them for 2-1/2 hours. However, no decrease in the severity of the toxic effect could be noted when the cooked peas together with the water in which they were cooked were fed at the 50 per cent level. Later the toxic principle was found to be water soluble and fairly stable to heat at the boiling point of water. In these

experiments the peas were soaked over night in distilled water and were then brought to the boiling point. After standing for 30 minutes the water was poured off. The peas were then covered again with distilled water and the extraction was repeated as before, five times. A final extraction was made with a large amount of water, this extract being discarded. The other combined extracts were concentrated to a small volume at 60 to 65° C. before an electric fan and were then dried on dextrin at the same temperature. When this was incorporated in the ration so that each 100 grams of ration carried an amount of extract equivalent to 100 grams of sweet peas, the same toxic effects were observed as when 50 per cent of sweet peas were fed. Furthermore, the extracted sweet peas fed at the 50 per cent level produced no apparent toxic effects after 11 weeks.

An attempt to prevent or correct the symptoms of lathyrism was made along the lines suggested by Mellanby (3) by vitamin additions. Cod liver oil did not protect the young animals from the toxic effect of the higher levels of sweet peas. In fact with 0.1 per cent of cod liver oil concentrate in addition to the 2 per cent of cod liver oil no decrease in the severity of the skeletal changes could be noted. Dried yeast was also without protective effect. However, the adult animals were protected to some extent by 2 per cent of cod liver oil at both the 50 and 25 per cent levels, which was apparent in the condition of both the femora and humeri. The bones of the animals receiving the cod liver oil were smaller, had fewer exostoses, and were more nearly of the same color as the controls.

It is noteworthy that so much growth was possible when no vitamin A was present in the diets other than that furnished by the sweet peas. With only 5 per cent of sweet peas in the diet normal growth was secured for 20 weeks. This indicates that the sweet peas used by us were unusually rich in vitamin A as compared with other seeds.

It is possible that the vitamin A in the sweet peas exerted a substantial neutralizing effect even without the addition of cod liver oil. It is suggested that future studies designed to determine the neutralizing effect of vitamin A be carried out with water extracts inasmuch as these can be readily obtained free from vitamin A. However, we believe that even with our present technic we discovered some prophylactic action with cod liver oil. The results seemed to be clean-cut with our adult animals.

#### SUMMARY

Lathyrism was produced in both young and adult rats by feeding diets which contained *Lathyrus odoratus*, the flowering sweet pea.

Growth of the young animals was retarded by sweet peas when they were fed as 80 per cent, 50 per cent and 25 per cent of the diet. However, normal growth was obtained for 20 weeks with 12.5 per cent and 5 per cent of sweet peas. Other symptoms of lathyrism noted were lameness, spinal curvature, sternal curvature, enlargement of the costochondral junctions, and malformation and abnormal red color of the long bones. Calcification was interfered with in young animals. The decided reddish color of the bones harmonized with a slight polycythemia and increased hemoglobin content of the blood. Hernias were also observed.

The young animals received no protection against the toxicity of the sweet peas from cod liver oil or cod liver oil concentrate, or from dried yeast. However, the adult animals were protected to some extent by the inclusion of 2 per cent of cod liver oil in the ration.

Cooking the sweet peas for 2-1/2 hours did not destroy their toxicity.

The toxic factor was extractable from the peas by water at the boiling point.

The sweet peas used by us proved to be a better source of vitamin A than any other seeds previously studied, since normal growth was secured on a diet in which 5 per cent of sweet peas was the only source of vitamin A.

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## THE VITAMIN CONTENT OF LICHENS\*

By

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THE native forage available to the herds of reindeer and musk-oxen maintained in the arctic regions of North America is by necessity limited chiefly to those plants which grow in the regions bordering on or beyond the arctic circle. The lichens are probably the most important group of plants available for grazing, particularly in the inland areas and during the winter season.<sup>1</sup> With the growth of the herds of reindeer and musk-oxen in Alaska has come the question of finding and selecting the best feeding areas for the herds. Out of these considerations of management has arisen the question of the nutritive value of the lichens. Recent studies (2) on the digestibility of feed by reindeer and musk-oxen have shown that the lichens yield moderately high digestibility coefficients.

An examination of the literature disclosed little or no information on vitamin values of lichens. On the other hand, Hadwen and Palmer (1) have described a number of diseases common in herds of reindeer, certain of which are suggestive of vitamin deficiencies. Dermatitis, keratitis, rickets, and other bone deformities are among those of possible dietary origin.

### THE LICHEN SAMPLES

The estimations of vitamin content have been confined to the vitamin A, vitamin B-complex (with partial differentiation of the B and G factors),

\* This study has formed a part of a coöperative investigation on nutrition problems relating to reindeer and musk-oxen by the Bureaus of Biological Survey and Animal Industry of the United States Department of Agriculture. The present study on the vitamin content of lichens was suggested by the Bureau of Biological Survey and the samples were obtained by L. J. Palmer in charge of the reindeer experiment station and grazing investigations of the Bureau of Biological Survey in Alaska. The work was conducted by the other authors named in the nutrition laboratories of the Bureau of Animal Industry at Beltsville, Maryland.

The authors wish to express their appreciation to Dr. W. B. Bell, of the Bureau of Biological Survey and to Dr. Paul E. Howe of the Bureau of Animal Industry for the suggestions they made relative to the conduct of the work.

<sup>1</sup> Hadwen and Palmer (1) have described the grazing conditions and the types of vegetation available in Alaska. They have also compiled a check list of Alaskan range plants.

and the vitamin D factors. Samples of lichens were supplied in 1929 and again in 1930 for the feeding tests. Two composite samples, designated as Series 1, were made up from samples of separate species for feeding tests during 1929. One sample comprised those species of short lichens which grow in dry areas and the other, the tall forms, from moist areas. The list of species follows:

Short growth type or dry site lichens	Tall growth type or moist site lichens
<i>Alectoria nigricans</i>	<i>Cetraria islandica</i>
<i>Alectoria ochroleuca</i>	<i>Cetraria cucullata</i>
<i>Cetraria nivalis</i>	<i>Cladonia alpestris</i>
<i>Cetraria hiascens</i>	<i>Cladonia gracilis elongata</i>
<i>Cladonia bellidiflora hookeri</i>	<i>Cladonia uncialis</i>
<i>Cladonia gracilis dilatata</i>	<i>Cladonia amaurocraea forma</i>
<i>Lobaria linita</i>	<i>Cladonia sylvatica sylvestris</i>
<i>Nephroma arcticum</i>	
<i>Parmelia physodes</i>	
<i>Peltigera species</i>	
<i>Stereocaulon tomentosum</i>	
<i>Thamnia vermicularis</i>	

Series 2 consisted of an additional pair of samples of short and tall lichens gathered at the experiment station at College, Alaska, in May, 1930. The sample of short growth lichens consisted largely of *Cladonia bellidiflora hookeri* and *Cladonia gracilis dilatata*. The other was made up of mixed species of tall growth lichens as in Series 1. The lichen samples were finely ground in order that the required amounts could be intimately incorporated into the basal diets. Both series were used in the tests on vitamin A and on the vitamin B-complex, while Series 2 was used in the tests reported on vitamin D.

#### EXPERIMENTAL PROCEDURE

##### *The Growth of Rats on Lichens-Containing Diets*

In preliminary tests it was found that rats did not readily consume large amounts of lichens when fed *ad libitum* separately from the basal diet. Incorporation of the lichens into a mixed diet permitted adequate food consumption when the short lichens were used, but not when the tall lichens were present at levels of 10 or 20 per cent. Results of these feeding tests are illustrated in the lower part of Figure 1. The diets were similar to those



at 10 and 20 per cent levels did not lower the food consumption or the rate of growth to any marked extent. In the case of the tall lichens, 5 per cent did not interfere with growth, while 10 per cent retarded growth during the first four weeks of the feeding period (the second month in the life of the rats) and caused cessation of gain in weight when reintroduced after a period on the 5 per cent level. Increase in the tall lichen content to 20 per cent caused fatal results in all cases. The fiber content of the tall lichens (Table I) was 34 per cent in the sample of Series 2. Since other feeds with an equally high fiber content have been fed without such pronounced ill effects, it appears probable that the fiber was not the harmful factor. Further discussion of the properties of extracts of lichens is given at a later point. The inability to feed large amounts of lichens, especially the tall form, has limited the scope of the present studies on rats.

TABLE I  
COMPOSITION OF SHORT AND TALL FORMS OF LICHENS USED IN FEEDING TESTS

Type of lichen	Water	Protein	Ash	Fat	Crude fiber	Nitrogen free extract
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
Short form, Series 1 average (5 species).....	13.4	6.0	3.4	3.7	15.4	57.9
Tall form, Series 1, average (8 species).....	12.5	2.1	1.8	2.3	32.3	49.1
Tall form, Series 2, as received	12.09	2.45	2.57	2.64	34.00	46.25

### *The Vitamin B-complex and Associated Factors*

Estimation of the vitamin B-complex was accompanied by supplemental tests on rats to determine whether vitamin B or vitamin G was present in measurable amounts. Young rats were individually fed a basal diet deficient in the vitamin B-complex. This diet (No. 149) consisted of: water and alcohol extracted casein, 20 per cent; dextrinized rice starch, 65 per cent; lard, 10 per cent; agar, 1 per cent; mineral mixture (Osborne and Mendel), 4 per cent; and cod liver oil fed separately in 0.2 cc. doses three times per week.

Following a short depletion period of one to two weeks on this diet, the lichens were incorporated into the basal diet by substitution of equal amounts of dextrin as follows:

	<i>per cent</i>
Diet 160—tall lichens, Series 1.....	3
Diet 161—short lichens, Series 1.....	3
Diet 162—tall lichens, Series 1.....	6
Diet 163—short lichens, Series 1.....	6
Diet 177—short lichens, Series 2.....	10
Diet 178—tall lichens, Series 2....	10

The growth curves of selected animals, typical of the results on each diet, are shown in Figure 1. Neither the 3 nor the 6 per cent levels of lichens was capable of maintaining growth any better than the basal diet (No. 149) known to be deficient in the vitamin B-complex. Even when autoclaved yeast,<sup>2</sup> a source of vitamin G, was added to the daily diet of the rats on the diets containing lichens their decline in weight continued, due, evidently, to the absence of even moderate or small amounts of vitamin B.

However, the substitution of 0.5 gram of rice polish for autoclaved yeast resulted in the resumption of gains. Although rice polish contains only small amounts of vitamin G as compared to its rich supply of vitamin B, observations indicated that the growth of the rats after the addition of the 0.5 gram dose was due to the presence of vitamin G in the rice polish rather than in the lichens. Ten per cent levels of the short and tall forms of lichens in Diets 177 and 178, respectively, were still inadequate as sources of the vitamin B-complex since three animals on each diet died within approximately the same time as control animals on Diet 149. Death occurred earlier on Diet 178 than on the complete diets previously described.

Tests on the vitamin G content of the lichens were also made by the use of a diet (No. 184) which contained white corn as a source of vitamin B. This diet differed from diet 149 in the substitution of 25 per cent of ground white corn for the lard and agar and for 14 per cent of dextrin.

Rats on this diet alone and supplemented with alkaline autoclaved yeast as a source of vitamin G are shown in Figure 1. Diets 185 and 186 contained 20 per cent of the short and tall types respectively of lichens of Series 2 in place of an equal percentage of dextrin. Growth curves of rats on these diets indicated that little, if any, vitamin G was present even in the small lichens.

Further evidence that the tall lichens were deficient in the vitamin B-complex was afforded by feeding tests on an extract prepared from the

<sup>2</sup> The yeast was adjusted to a pH of 9.0 with sodium hydroxide, autoclaved for two hours, then neutralized with hydrochloric acid and dried at room temperature with a fan. The daily dose was 0.4 gram.



residue after extraction of the lipids for vitamin A tests (see page 449). The fat-free residue was extracted with 25 per cent alcohol, concentrated by vacuum distillation, then spread on dextrin and dried. Two animals fed Diet 149 plus doses of this extract equivalent to 1 gram per day of lichens, which was increased on the 35th day to 2 grams, gradually lost weight and both died on the 47th day. The food consumption was 102 and 127 grams respectively for the entire period, which was lower than for the control animals on Diet 149 used in the present experiment. It appeared evident that this lichen extract was distinctly unpalatable like the whole lichen.

### *Vitamin A*

The vitamin A estimations were made by the therapeutic method. Young rats were placed in individual cages at the age of 21 or 22 days and fed a vitamin A-deficient diet (No. 158) which consisted of: alcohol extracted casein, 18 per cent; dextrinized rice starch, 72 per cent; agar, 1 per cent; irradiated dry yeast, 5 per cent; mineral mixture, (Osborne and Mendel) 4 per cent. When further gains in weight ceased on Diet 158, the diets containing lichens were substituted. This change in diet was made, in the usual case, between the thirty-seventh and fortieth days. Various levels, ranging from 2 to 20 per cent of the short and from 2 to 10 per cent of the tall lichens were substituted in Diet 158 in place of proportionate quantities of dextrin.

The results on 38 rats are shown in Table II. The animals are grouped in the table according to percentage of lichens in the therapeutic diet. In a number of cases, animals fed the same level of the first and the second consignments of lichens are grouped together. The gains in weight during the therapy period are given in the table at the end of four, six, and eight weeks. In those cases where the animals died after four or six weeks on test feeding, the gains or losses in weight and the average daily feed consumption are given for the periods which the animal survived. The condition of the animals at the time of death or at the close of the experiment is also given.

The short lichens fed at a 2 per cent level relieved the avitaminosis condition in part and enabled three of the five rats to survive the therapeutic period. Two per cent of tall lichens were without benefit to three animals. All animals on the 5 per cent level of short lichens survived the experiments, showed only minor symptoms of vitamin A deprivation, and gained an average of 56 grams on a calculated daily consumption of approximately 0.5 grams of short lichens. This dosage was more than sufficient to

furnish one vitamin A unit (4) based on an average gain of 3 grams per week.

One, of a group of six animals, survived the eight-week test period on the 5 per cent level of tall lichens. The survival period was longer than on the 2 per cent level, which may be taken to indicate that the tall lichens contain at least small amounts of vitamin A. However, all five animals on a 10 per cent level of tall lichens died within four weeks, due primarily to a lack of vitamin A.

A 200-gram sample of tall lichens was dried at reduced pressure in an atmosphere of carbon dioxide for two hours at 70°C., then extracted with absolute alcohol. The alcoholic extract was removed from the flask at frequent intervals during the early part of the extraction in order to avoid long continued heating. At the end of six hours, the alcohol was entirely drained off the lichens and ether added. The extraction was continued for eight additional hours. The combined extracts were concentrated under reduced pressure and finally taken up in a hydrogenated vegetable fat free of vitamin A. This concentrate was fed to three rats to test its vitamin A potency. One rat received the equivalent of 0.5 gram and two others of 1.0 gram of lichens daily which corresponded roughly to the amounts on the 5 and 10 per cent levels respectively. This absolute alcohol-ether extract did not cause a pronounced decrease in food consumption such as was observed on an equivalent amount of unextracted tall lichens. The lichen extract also failed to arrest the development of characteristic symptoms of avitaminosis-A. It appears evident that the tall lichens contained considerably less vitamin A than the short lichens.

#### *Vitamin D*

The estimation of the antirachitic properties of lichens was carried out by the therapeutic method. Young rats were fed for four weeks on the Steenbock-Black rachitogenic ration which consists of yellow corn meal, 76 per cent; wheat gluten, 20 per cent; calcium carbonate, 3 per cent; and sodium chloride, 1 per cent (5).

At the beginning of the fifth week, the therapeutic diet was substituted. The lichens of Series 2 replaced equal amounts of corn in the rachitogenic diet at levels of 2, 5, 10, and 20 per cent. The eight days on therapy were followed by an additional day on the rachitogenic diet to allow for further assimilation and action of the antirachitic principle. At the end of this period the animals were weighed, killed, and the rear leg bones removed for ash analysis and line tests. The tibiae were placed in 10 per cent

TABLE II  
THE RESULTS OF FEEDING TESTS WITH RATS ON THE VITAMIN A CONTENT OF LICHENS

Lichens Type, series and per cent	Sex and initial weight	Gain deple- tion	Condition and gain on therapy			Daily feed consumption	
			4 weeks	6 weeks	8 weeks	Deple- tion	Therapy
Basal diet 158 with- out supplement	grams	grams	grams	grams	grams		
	37 ♂	144	Died 19 days (++++)*			12.6	
	40 ♂	137	-89 died 28 days (+++++)			11.6	
	31 ♀	72	Died 19 days (+++++)			8.3	
	31 ♂	52	" 19 " (++++)			6.4	7.5
	35 ♀	35	3	-17	-36 (+++++)	10.6	
Short (1) 2-10 " (2) 2	45 ♂	152	-40	-17	-16 (+)	12.5	11.5
	33 ♂	73	16	23	Died 47 day (+++++)	6.8	
	27 ♀	53	17	4	" 42 " (+++++)	6.6	
	35 ♀	70	20	-2	14 (+)	8.7	7.7
	34 ♀	66	20	25	26 (+)	7.7	8.1
Tall (1) 2	36 ♂	113	Died 27 days (++++)			9.8	
	44 ♀	75	" 19 " (++++)			9.4	
	42 ♂	106	" 19 " (++++)			10.9	
Short (1) 5 " (2)	38 ♂	112	49	63	69 (-)	11.9	12.7
	33 ♂	62	39	72	59 (+)	7.4	9.6
	40 ♂	26	60	86	69 (+)	5.1	10.2
	33 ♀	67	30	41	42 (+)	10.4	9.3
	29 ♀	18	29	45	43 (+)	7.4	7.2

Tall (1) 5	45 ♂ 32 ♀	166	-60 Died 28 days (++++)	-76 Died 40 day (++++) " 42 " (++++) " 33 " (++++) " 37 " (++++)	-91 (++)	13.4 9.8 8.4 9.7 9.8 6.9	10.2 5.9
" (2)	35 ♂ 36 ♂ 30 ♀ 30 ♀	70 99 64 49	-25 -36 -34 -19				
Short (2) 10	39 ♂ 33 ♂ 34 ♀	60 76 58	61 51 51	87 91 73	94 (-) 111 (-) 81 (-)	10.8 6.8 8.1	12.2 13.2 7.2
Tall (2) 10	33 ♂ 31 ♂ 28 ♂ 29 ♀ 30 ♀	62 81 63 63 38	Died 8 days (++++) " 26 " (++++) " 12 " (++++) " 19 " (++++) " 9 " (++++)			9.1 8.9 6.7 9.5 6.2	
Diet 158 plus alcohol 0.5 grams† ether-extract tall lichens 1.0 gm.	37 ♂ 36 ♂ 35 ♂	134 131 59	-59 -76 Died 20 day (++++)	Died 40 days (++++) Died 31 days (++++)		10.1 10.4 6.1	

\* The symbols -- and + represent gradings of the condition of the animals relative to stages of vitamin A deprivation from a grade of -- for nearly normal, then + to ++++ for advancing stages of vitamin A deprivation.

† The quantities given are equivalents of lichens as the daily dose, that is, such quantity of extract was added as the daily dose as was obtained from 0.5 to 1.0 grams of lichens.

TABLE III  
ANTIRACHITIC VALUE OF SHORT AND TALL TYPE LICHENS AS DETERMINED BY  
FEEDING EXPERIMENTS WITH RATS

Type and per cent of lichens in curative diet	Depletion period 28 days		Curative period—8 days					
	Total gain	Daily food	Total gain	Daily food	Daily doses lichens	Line test	Bone analysis	
							Ash	A/R
	grams	grams	grams	grams	grams		per cent	ratio
Negative controls	19	7.9				—	36.9	.59
	15	7.0				—	39.8	.69
	20	8.7				—	35.1	.54
Short type lichens 5 per cent	19	5.4	6	7.5	.38	—	38.7	.63
	11	6.6	5	7.0	.35	—	32.0	.47
	24	8.4	2	7.0	.35	+	38.6	.63
	15	8.3	2	2.1	.11	++	42.6	.73
10 per cent	20	6.9	—6	8.0	.80	++	44.8	.81
	20	7.6	—2	5.1	.51	+	35.4	.55
	19	6.0	3	9.0	.90	++	43.1	.76
	13	9.0	1	9.6	.96	+	43.6	.77
20 per cent	10	6.1	—2	6.8	1.35	++	45.6	.84
	18	6.5	—3	8.3	1.65	++	36.6	.58
	4	7.7	5	10.1	2.02	++	43.1	.76
Tall type lichens 2 per cent	11	7.8	—7	6.9	.14	++++	44.9	.81
	4	7.7	—5	6.8	.13	+++	48.5	.94
	9	7.7	—4	6.8	.13	++	46.9	.88
5 per cent	19	7.6	0	6.0	.30	++++	44.3	.81
	16	8.1	3	6.6	.33	+++	37.1	.59
	30	7.4	4	7.4	.37	+++	38.3	.62
	18	7.9	11	6.2	.31	+++	48.2	.93
<i>ad. lib.</i>	21	6.9	—3	3.8	.28	—	38.5	.63
10 per cent	15	8.0	—10	6.4	.64	++++	43.5	.76
	23	8.4	—6	4.8	.47	++++	40.0	.67
	19	7.5	—8	7.6	.76	+++	47.7	.91
20 per cent	18	6.3	—18	3.5	.70	++++	43.4	.77
	22	7.1	—10	5.0	1.00	++++	45.5	.84

formalin and line tests made according to the technic outlined by Bills, Honeywell, and McNair (6). Photomicrographs of the "line tests" have

greatly facilitated interpretation of the readings (7). The femurs were carefully cleaned of adhering flesh, dried, weighed, crushed with a haemostat, placed in a paper capsule, and extracted in a Soxhlet apparatus with alcohol and then ether for 12 to 14 hours each. The residue was dried, weighed, and ashed. The percentage of ash based on the extracted bone weight and the A/R ratio (ash to organic residue) as proposed by Chick, Korenchevsky, and Roscoe (8) were calculated.

The results are given in Table III. The animals used as negative controls, which were killed at the end of the four-week depletion period, all showed uniformly negative line tests. Of the groups on the three levels of short lichens, the one on 10 per cent showed definite healing in all cases.

On the 2 and the 5 per cent levels of tall lichens, definite healing was fully evident as shown by the line test readings. Above these levels there is evidence in certain cases of abnormal healing, presumably due to partial starvation superimposed upon the healing induced by the lichens. Analyses on the tall lichens showed 0.13 per cent and 0.03 per cent respectively of calcium and phosphorus. Accordingly, no additional calcium was required in the diets to maintain the 5:1 ration of calcium to phosphorus.

The results indicate that the short lichens induced healing of rachitic rats at a 10 per cent level, while the tall lichens were somewhat more effective at a level of only 2 per cent. In this connection it is of interest to note that Zellner (9) has reported the isolation of ergosterol in a lichen, *Peltigera canina*.

#### SUMMARY

Feeding tests with rats failed to show the presence of the factors constituting the vitamin B-complex in either short or tall type lichens at the levels at which it was possible to feed them.

Tests for the vitamin A response showed that 5 per cent of short lichens added to the basal diet during a curative period permitted a moderate rate of gain. No vitamin A could be demonstrated in the tall lichens, either when fed whole or as an alcohol-ether extract at levels up to or equivalent to 10 per cent of the diet.

The tall lichens apparently possessed greater antirachitic properties than the short lichens. Curative tests showed a marked difference at a 5 per cent level.

The tall lichens were not palatable to rats and few survived for any extended time when fed at levels greater than 10 per cent. The short lichens were well tolerated at the levels fed.

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## EFFECT OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

By

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THE effect of eating cranberries on human blood alkali reserve has not been reported in the world's scientific literature. Aside from the single experiment reported by Blatherwick and Long (1) in 1923, there are likewise no data bearing on the effect of cranberries on urinary acidity. These investigators found both prunes and cranberries when ingested in large quantities (about 300 grams per day) produced marked increases in both the titratable and organic acid acidities of the urine. The prunes contained 0.149 per cent benzoic acid and produced about double the acidity of a similar weight of cranberries with a benzoic acid content of .096 per cent. These amounts of benzoic acid being far too small to account for the large amounts of hippuric acid found in the urine, Blatherwick and Long conclude that the principal source of hippuric acid in the urine is probably quinic acid. Lücke (10) observed that the excretion of hippuric acid was elevated after the ingestion of many vegetables and fruits, especially cranberries. Quick (12) agrees with Blatherwick and Long that quinic acid is the substance present in fruits which is responsible for the increased excretion of hippuric acid. Kohman and Sanborn (9) have recently isolated quinic acid from both cranberries and prunes. There is no direct, accurate method of determination. Quick (12) demonstrated that quinic acid in the human organism was first transformed to benzoic and finally to hippuric acid. The transformation takes place more slowly than with benzoic acid and elimination is incomplete even after 24 hours. In general, elimination of 80 per cent or more of the benzoic acid occurs within six hours after ingestion.

In the conjugation of benzoic acid with glycine to form hippuric acid, there is no evidence of a deficiency of glycine to bring about this change unless abnormally high doses of benzoic acid are ingested. The glycine utilized for the conjugation normally is not derived entirely from the diet but is synthesized as needed. It is presumed though not proved that glycine is synthesized largely by the liver. Swanson (13) concludes that glycine is probably synthesized from the constituents which are normally converted



to urea. Griffiths and Lewis (7) demonstrated that the rate of excretion of hippuric acid was decidedly increased by the administration of glycine alone with the benzoic acid.

Other substances which have been shown to form hippuric acid in the body are benzaldehyde, cinnamic acid, and toluene. The first two of these substances occur normally as glucosides in various natural food products but are not known to occur in cranberries.

#### OBJECT OF RESEARCH

The object of the present research was to determine in human subjects the effect of ingested cranberries on the composition of the urine and on the blood alkali reserve.

#### METHODS

Six male human subjects, from 22 to 27 years of age, were used in these experiments. They are designated as P, R, N, J, C, and W and were given a daily basal diet consisting of:

Graham crackers.....	300 grams
Milk (3 pints).....	730 grams
Iceberg lettuce.....	120 grams
Heavy cream ( $\frac{1}{2}$ pint).....	225 grams
Eggs (2).....	140 grams
Apple (1).....	150 grams

In the second experiment (Table II) subjects N and J followed this diet with the exception that they ate no apples. In the third experiment (Table III) the basal diet was followed with the exception that Subject C used no cream and ate two apples a day. Subject J ate two apples a day in place of one. The basal diet was eaten for several days or until the body reached a metabolic equilibrium, after which cranberries were eaten in the desired amounts.

Three experiments were conducted. The first afforded a means of observing the effect of the ingestion of large amounts of cranberries and was made on Subjects P and R (Table I). The second was run in an effort to determine the rate with which the body recovered from the effects produced by eating large quantities of cranberries. Subjects N and J were used in this experiment the results of which are tabulated in Table II. The third experiment was made on Subjects C, W, P and R using small normal servings of cranberries (Table III).

The Kingsbury and Swanson (8) hippuric acid technic was used and found very satisfactory. Urea nitrogen was determined by the urease

TABLE I  
EFFECT OF LARGE DOSES OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

Date July, 1931	Sub- ject	Volume cc. per 24 hrs.	Sp. gr.	pH	Grams in 24 hours							Blood* alkali reserve cc.	Diet	
					Nitrogen		Creat- inine	Phos- phorus	Hippu- ric acid	Uric acid	Tit. acid			Org. acid
					Urea	Am- monia								
5 day av. 14-18	R	871	1.022	6.26	10.58	0.45	1.01	1.30	0.80	0.33	397	446	Basal	
	P	1599	1.012	5.96	10.70	0.74	1.30	1.08	0.78	0.31	474	517		
19	R	710	1.023	6.1	10.44	0.20	0.73	1.20	1.57	0.18	465	558	Basal+100 grams cranberries	
	P	1200	1.015	5.8	12.36	0.26	0.93	1.00	1.64	0.28	478	566		
20	R	880	1.021	6.1	9.56	0.44	0.92	1.33	2.49	0.29	456	608	Basal+150 grams cranberries	
	P	1170	1.015	5.7	9.46	0.54	1.04	0.96	2.46	0.33	451	665		
21	R	775	1.020	6.2	8.32	0.46	0.80	0.93	3.00	0.24	363	600	Basal+200 grams cranberries	
	P	1945	1.012	5.8	10.56	0.94	1.30	0.89	3.88	0.31	451	702		
22	R	1000	1.018	6.1	8.66	0.44	1.05	1.16	3.16	0.32	442	614	Basal+250 grams cranberries	
	P	1065	1.018	5.6	8.36	0.52	1.11	0.91	3.40	0.39	443	682		
23	R	1080	1.015	5.8	7.24	0.58	0.87	1.09	4.35	0.22	492	694	Basal+300 grams cranberries	
	P	1840	1.010	6.2	7.94	1.06	1.23	0.74	4.48	0.17	398	764		

\* CO<sub>2</sub> per 100 cc. plasma.

TABLE II  
EFFECT OF CRANBERRIES ON URINARY AND BLOOD ACIDITY. RATE OF RECOVERY

Date Aug. 1931	Subject	Volume	Sp. Gr.	pH	Grams in 24 hours					Alkali** reserve plasma cc.	Remarks
					Nitrogen		Hipp. acid	Tit. acid	Org. acid		
					Amm.	Urea					
Av. 3-4	N	1260	1.015	6.6	0.54	10.03	0.82	282	526	81.4	Basal diet
	J	610	1.030	5.75	0.44	9.83	0.93	469	585	62.5	
5	N	1190	1.015	6.3	0.60	11.00	5.52	354	649	51.0	(after 2 hrs.) Basal diet plus 254 grams cranberries
	J	430	1.030	6.2	0.48	8.72	0.90*	292	419	49.1	
6	N	1015	1.018	6.5	0.52	8.88	1.75	360	732	34.3	(12 hrs.) Basal diet
	J	580	1.030	5.8	0.52	8.78	3.15	460	647	44.2	
7	N	1635	1.012	6.6	0.64	9.14	1.45	307	557	77.3	(36 hrs.) Basal diet
	J	540	1.033	5.9	0.43	9.15	0.74	462	530	72.6	
8	N	1000	1.018	6.6	0.50	8.60	0.93	282	546	73.6	(60 hrs.) Basal diet
	J	605	1.028	6.2	0.55	9.16	0.87	363	590	67.6	

\* On this date Subject J failed to collect the early morning sample of urine which undoubtedly contained practically all the hippuric acid for that day as the cranberries were eaten about 6 o'clock the evening before.

\*\* CO<sub>2</sub> per 100 cc. plasma.

method as described by Folin and Youngburg (6), uric acid by the Christman-Ravwitch modification of the Folin and Franke method (2), and organic acids by the Van Slyke and Palmer technic (15). Ammonia N was determined by the Folin and Bell method (5); creatinine by Folin's method (4) and phosphorus by the Fiske and Subbarow method (3).

The figures in the tables are expressed in terms of grams per 24 hours in all cases except titratable acidity and organic acids which are reported as cubic centimeters of one-tenth normal acid in 24 hours. The alkali reserve is expressed as cubic centimeters of carbon dioxide in 100 cubic centimeters of blood plasma. In the last two experiments it was deemed unnecessary to determine creatinine, phosphorus, or total nitrogen, since these values showed no particular variation.

Blood samples were taken at varying hours following the consumption of cranberries. The experimental day began and ended at 8 a.m. Cranberries were eaten at noon and evening in the first experiment, at 6 p.m. in the second and at noon in the third experiment. Urine samples were kept in the refrigerator to prevent bacterial decomposition.

Both Howes and Early Black cranberries were used, usually in the form of whole or strained cranberry sauce. However, all results are calculated to weight of fresh fruit.

#### DISCUSSION

Table I contains detailed data on the various constituents of urine as well as on blood alkali reserve.

In general the data on volume, specific gravity, pH, creatinine, and phosphorus are of little significance and show no important changes during the course of the experiments. Both titratable and organic acids are increased following consumption of large quantities of cranberries. Doses of 100 grams or more of cranberries in a 24 hour period increased the titratable and organic acids in the urine not over 15 per cent in any case.

Hippuric acid gave the best gauge of the quantity of cranberries ingested, the quantity found in the urine being directly proportional to the weight of cranberries eaten. The only exception is found in Table III where small dosages of cranberries (22 to 54 grams) daily showed but slight increase in hippuric acid.

Since upon analysis the cranberries showed from 0.05 to 0.1 per cent benzoic acid, the balance of the hippuric acidity must have originated in some other substance viz., quinic acid. Thus indirectly, we find that cranberries contain from 0.5-0.8 per cent of quinic acid. Since quinic acid acts in the same manner as benzoic, in forming hippuric acid in the human

body, we can thus account for the increased acidity of the urine following the eating of cranberries.

Even small amounts of cranberries, Table II, 22 to 54 grams, equivalent to from 2 to 5 ounces of cranberry sauce, produced slight increases in urinary acidity, though from a physiological or health viewpoint this slightly increased acidity is without significance. These amounts probably represent average servings of cranberries. Table II shows that the recovery of urinary acidity to normal after eating large doses of cranberries may be delayed for 48 hours though a large part of the hippuric acid is voided during the first 24 hours after eating the cranberries. This experiment confirms Quick's (12) results which show benzoic acid is eliminated from the body within a very few hours after ingestion while quinic acid is eliminated much more slowly.

Since in the determination of organic acids, hippuric acid is also included, the same variations occur in this determination as were just discussed for hippuric acid. The hydrogen ion concentration of the urine was usually slightly increased by eating cranberries, the increase being approximately 0.2 for large doses of cranberries. Uric acid appears to decrease slightly in the urine. Quick (12) explains this decrease in uric acid excretion on the basis that all the glycine present in the kidney may be withdrawn to detoxify the benzoic acid when large amounts are fed.

The ammonia content of urine increased a little after eating cranberries. The urea was perceptibly decreased, the nitrogen which normally is eliminated as urea may be required to form the glycine which in turn is necessary for the conjugation and elimination of benzoic acid as hippuric acid.

Cranberries are thus seen to increase both titratable and organic acidities, hippuric acid, and ammonia, while urea nitrogen, uric acid, and pH are decreased.

#### *Blood Alkali Reserve*

Alkali reserve of the blood was determined by means of the Van Slyke apparatus (14). The carbon dioxide gas, from the bicarbonates, is liberated from a measured sample of blood plasma and its volume carefully determined. Duplicate and sometimes triplicate determinations were made. Individuals vary a great deal in their blood alkali reserve and a value that is normal for one person may be quite abnormal for another. However, by using the same individual in feeding tests involving alkali reserve, comparative and conclusive results may be obtained.

The data in Tables I and II are largely self-explanatory. Large doses of cranberries have a marked effect in decreasing the blood alkali reserve.

EFFECT OF SMALL AMOUNTS OF CRANBERRIES ON URINARY ACIDITY AND BLOOD ALKALI RESERVE

Date Aug., 1931	Sub- ject	Vol. urine 24 hrs.	Sp. gr.	pH	Grams in 24 hours					Blood* alk. res. (Plasma) cc.	Diet
					Nitrogen		Hipp- uric acid	Titra- table acid	Org. acids		
					Urea	Amn.					
Av. 16-18	C W	795 1218	1.021 1.016	6.2 6.6	9.87 8.88	.413 .577	1.12 1.35	351 291	527 544	75.5 77.6	Basal Basal
19	C W	870 1660	1.018 1.012	6.4 6.7	9.58 10.25	.52 .72	1.24 1.45	332 267	— 673	73.6 73.9	Basal+22 grams of cranberries (19 hrs.) (5 hrs.)
20	C W	960 1215	1.020 1.021	6.6 6.2	9.96 8.70	.46 .60	1.21 1.53	280 408	— 607	74.2 78.8	Basal+22 grams of cranberries
21	C W	1115 920	1.018 1.020	6.6 6.3	9.45 9.17	.55 .49	1.33 1.20	276 363	475 —	79.5 79.5	Basal+22 grams of cranberries (12 hrs.) (2 hrs.)
June, 1932					Uric acid						
Av. 14-15	R P	893 1420	1.024 1.015	6.65 6.2	10.4 9.8	.46 .43	1.01 0.90	269 366	516 572	71.7 69.3	Basal Basal
16	R P	940 1535	1.018 1.015	6.0 6.2	10.7 10.9	.49 .44	1.07 1.29	425 435	504 589	67.4 75.2	Basal+32.7 grams of cranberries (4 hrs.) "
17	P	1250	1.025	6.2	11.2	.42	1.63	400	500	73.6	Basal+32.7 grams of cranberries "
18	P	1680	1.015	6.5	9.9	.48	1.20	320	408	75.5	Basal+54 grams of cranberries "
19	P	1630	1.020	6.1	10.4	.41	1.77	350	462	73.1	Basal+54 grams of cranberries "
20	P	1480	1.020	6.2	10.5	.41	0.84	348	420	73.1	Basal "

\* CO<sub>2</sub> per 100 cc. plasma.

Quantities of cranberries varying from 100 to 254 grams (equivalent to 9–24 oz. of cranberry sauce) reduced the blood alkali reserve from 30 to 60 per cent. This degree of acidosis may be considered mild to moderate.

As shown in Table III, smaller amounts of cranberries, 22 to 54 grams, corresponding to 2–5 oz. of sauce, produced no demonstrable acidosis. It thus appears that the blood buffer substances are able to maintain the alkali reserve and  $pH$  even in the presence of moderate amounts of free acids. Though cranberries in normal servings do not lower the blood alkali reserve, they apparently do not leave an alkaline residue in the body in spite of the slight alkalinity of the ash (11).

#### SUMMARY

When cranberries were fed in amounts of 100–300 grams daily to normal young men the following observations were made as to the composition of the urine:

1. The titratable acidity, organic acids, hippuric acid, hydrogen ion concentration, and ammonia were increased, while uric acid and urea nitrogen were slightly decreased.

2. The amount of hippuric acid recovered in the urine was roughly proportional to the weight of cranberries eaten.

3. The hippuric acid was largely voided over a 24 hour period following the ingestion of cranberries.

4. The origin of most of the hippuric acid in the urine is presumably quinic acid or a glucoside which produces quinic acid upon hydrolysis. The cranberries used contained only 0.05 to 0.09 per cent benzoic acid, an amount totally inadequate to account for the large amounts of hippuric acid recovered in the urine.

5. By measurement of the hippuric acid content of the urine and the benzoic acid content of the cranberries ingested, the conclusion is reached indirectly that cranberries contain from 0.5 to 0.9 per cent quinic acid in free or combined form.

6. Two to five ounces of cranberry sauce equivalent to from 22 to 54 grams of fresh cranberries produced only very slight increases in urinary acidity. These quantities of cranberry sauce probably represent average servings.

7. The decrease in urea nitrogen in the urine is probably due to the additional quantities of glycine required for the conjugation of the benzoic acid in the body. The glycine may make use of nitrogen which would otherwise be voided in the urine as urea.

8. Large quantities of cranberries decrease the carbon dioxide combining capacity of the blood, the decrease being in general proportional to the quantity of berries eaten. The usual decrease after eating 100–300 grams of cranberries was 30 to 60 per cent and produced a mild to moderate acidosis.

9. When normal quantities of cranberries were eaten, no decrease in blood alkali reserve was found. Thus two to five ounces of cranberry sauce, equivalent to 22 to 54 grams of fresh cranberries, produced no demonstrable decrease in blood alkali reserve.

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## THE STIMULATING ACTION OF COPPER ON ERYTHROPOIESIS\*

By

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ALTHOUGH numerous investigations on the effect of copper in the presence of iron on the cure and prevention of nutritional anemia have been made during recent years, few workers have studied the effect of copper *without iron* on animals receiving a basal diet of milk. Among these Titus and Hughes (7), Waddell, Steenbock, and Hart (9), and Keil and Nelson (1) have followed only the hemoglobin in such studies, while Schultze (4) has made simultaneous erythrocyte counts and hemoglobin determinations. Before the latter paper appeared, the investigation herein reported on the effect of copper without iron on both hemoglobin and erythrocytes had been undertaken in our laboratory (5) incidental to certain investigations with evaporated milk.

### EXPERIMENTAL

Albino rats were taken from their mothers when 21 days old, placed in all-glass cages, and kept on a diet of evaporated or raw milk, the latter supplemented with copper in some cases, as indicated under experimental results.

The cage was essentially that described by Underhill, Orten, and Lewis (8), although some modifications of design have been made. Short glass rods long enough only to separate the tubing which constitutes the floor were employed in place of the glass-tube side walls of the original cage. For the side walls, double strength window glass measuring  $9\frac{1}{4} \times 27\frac{1}{2}$  inches was used. These were held together with strips of adhesive tape measuring  $1 \times 18$  inches. The same size glass was used for the top of the cage, the wooden frame being omitted. To prevent movement, the top was held in place by U-shaped glass tubing wide enough to span the width of the cage. The solid glass sides served to hold down the glass tubes used as flooring, thus eliminating the long glass tubes employed for this purpose in

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the original design. This arrangement also permitted the glass partition to rest directly upon the wooden frame, thereby making unnecessary the use of an extra glass tube alongside the partition to prevent contact of rats in neighboring compartments. As seen in the illustration (Fig. 1), the partitions are held in place by long glass rodding uprights. This cage can be taken apart and cleaned very quickly.

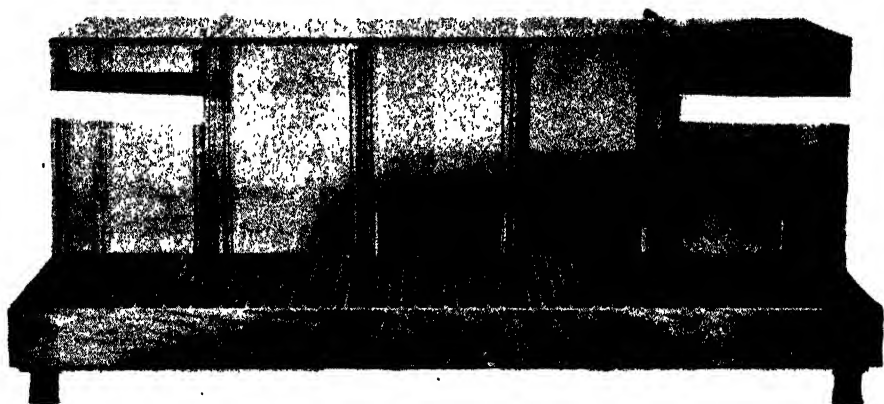


FIG. 1.—Modified glass cage.

The raw milk used was collected directly from the cow into wide-mouthed jars, a worker from the department supervising each collection. The evaporated milk, that purchasable on the open market, was made up as required from the large (14 1/2 ounce) can by dilution with an equal amount of water distilled from glass, and was kept in wide-mouthed glass jars. Both the raw milk and the diluted evaporated milk were kept in the refrigerator. The halves of standard Petri dishes used for feeding were washed in soap and water, placed in approximately 10 per cent nitric acid for 24 hours, then rinsed in water distilled from glass, and finally allowed to drain and to dry spontaneously. The milk was transferred to the feeding dishes by one-ounce "Asepto"<sup>1</sup> syringes, which were easily used and cleaned.

The copper was fed as copper sulfate in an aqueous solution of such strength that 0.5 cc. contained 0.25 mg. of copper, or, if less copper was

<sup>1</sup> Becton, Dickinson, and Company, Rutherford, New Jersey.

used, 0.025 mg. It was prepared from C.P. copper sulfate recrystallized several times from redistilled water.

The animals were weighed weekly, and hemoglobin determinations and erythrocyte counts were made biweekly at first, then weekly. The blood was obtained from the young rat by clipping the tail with a safety razor blade held in a patented holder. The tail was cauterized with a long, narrow strip of window glass, one end of which had been heated in a gas flame. As the animals became larger, it was possible to obtain blood by nicking one of the vessels of the tail. If bleeding did not stop quickly, a rubber band was used as a tourniquet by wrapping it around the tail a couple of times and holding it in place with an artery clamp.

For blood counts the regular Thoma diluting pipette was used, and Hayem's solution served as the diluent. The count was made in a Levy-Hauser counting chamber. The earlier hemoglobin determinations were made with the Hellige double glass standard Sahli apparatus. Simultaneous determinations made with the Bausch and Lomb Newcomer hemoglobinometer checked the Sahli readings within narrow limits. Later, a technic for the Newcomer apparatus providing greater speed and convenience with no loss in accuracy was used as follows: Five cc. of N/10 HCl were run from a burette into each of the required number of test tubes. The tubes were then closed with rubber stoppers and placed in a metal test tube rack. Each blood was collected in the regular Newcomer pipette and expelled into one of the test tubes, the pipettes being rinsed a few times by drawing up the acid and expelling it. The tube was shaken to mix the contents, and then replaced in the rack. The used pipette was washed in water and alcohol, and attached to a vacuum, where it dried in a few minutes and was ready for use again. The test tubes were allowed to stand for an hour before being read from the same source of artificial light, with the instrument placed the same distance from the light each time. This technic was convenient and accurate, and permitted the use of a minimum number of pipettes.

## RESULTS

The rats (5 animals) which were fed raw milk alone showed a sharp fall in hemoglobin and red cell count. In contrast the hemoglobin of the rats (5 animals) given diluted evaporated milk fell less rapidly, while the erythrocytes increased. These results, shown graphically in Chart 1, indicate that the evaporated milk contains an erythropoietic agent not found in raw cow's milk.

Since the milk comes in close contact with copper during the manufacturing process employed in the majority of evaporated milk plants, it seemed possible that copper absorbed by the milk might be the responsible agent for the erythropoiesis. Supplee and Bellis (6) in 1922 found that, when milk was passed through tin-lined copper pipes from which the tin was worn, the copper content increased as much as 2.0 mg. per liter.

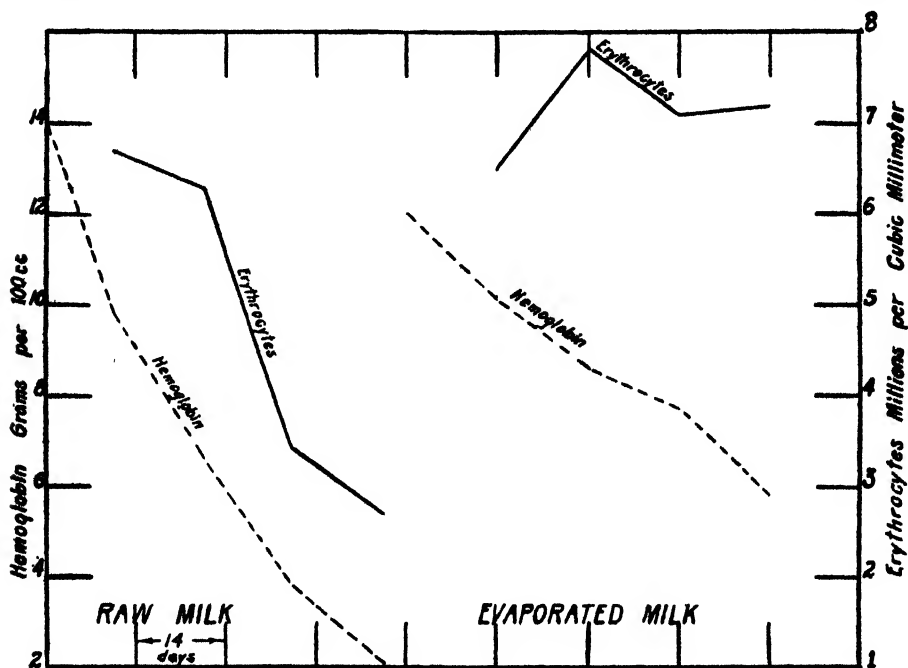


CHART 1.—Composite curves (average of 5 animals) showing the comparative effect on the hemoglobin and the erythrocyte count of feeding exclusive diets of raw milk and evaporated milk, respectively, to rats.

Rice and Miscall (3) reported that the amount of copper taken up by milk increased with the amount of copper surface to which the milk was exposed and with the length of exposure. Since these reports thus indicated that there would be a significant increase of copper in processed milk, an investigation was undertaken to determine if this metal was the causative factor in the maintenance of the red cell count when evaporated milk was fed. Consequently, 0.025 mg. of copper, the amount employed most often in nutritional anemia studies, was fed daily to each of a group of normal rats (6 animals) receiving raw milk as the basal diet. The results are shown in Chart 2. The hemoglobin fell gradually, but the number of erythrocytes

at the end of two weeks remained unchanged. During the next two weeks there was a slight increase in the red cell count followed by a gradual decrease in successive periods. The relatively slow fall in the erythrocyte count, as compared with that obtained when raw milk without copper is fed, indicates that copper plays a rôle in erythropoiesis.

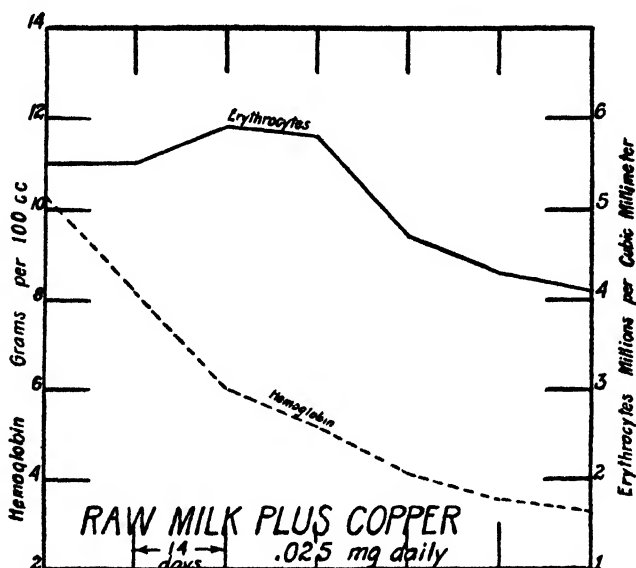


CHART 2.—Composite hemoglobin and erythrocyte curves (average of 6 rats) showing the effect of copper when added as the sole supplement to raw milk in maintaining the erythrocyte count at a relatively high level.

To confirm this supposition, an experiment was planned to see if copper would have an actual stimulating effect by increasing the red cell count of anemic rats. For subjects of these experiments, rats (4 animals) were fed raw milk alone until they became markedly anemic. After six weeks, when the red cells averaged 3,482,000 per cu. mm. and the hemoglobin, 3.4 gm. per 100 cc., 0.25 mg. of copper (ten times the previous dose) was given daily as a supplement to the milk diet. Chart 3 shows that during the next five weeks the erythrocytes increased to 5,725,000 per cu. mm. while the hemoglobin only rose to 4.75 gm. per 100 cc. Thus, it is evident that copper has an erythropoietic action independent of any influence it may have in the presence of iron on hemoglobin regeneration.

In the work of Schultze, to which reference has been made, larger amounts of copper (2 mg. daily), when given to anemic rats, produced an increase in the red cell count with little, if any, effect on the hemoglobin.

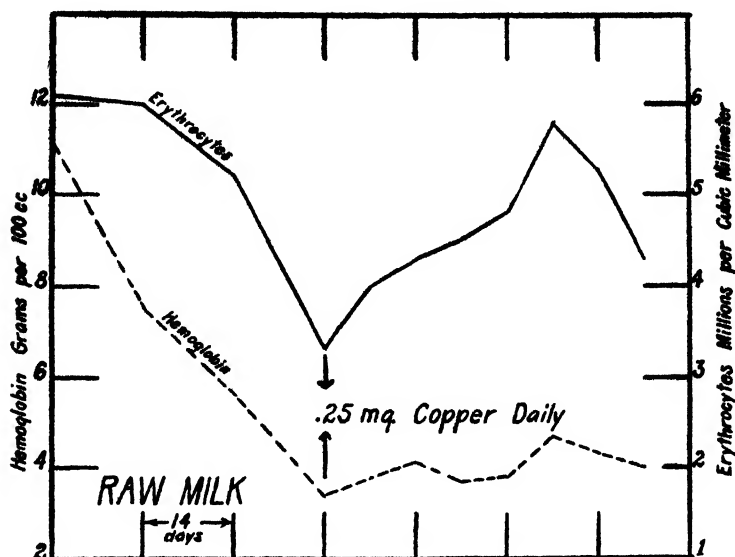


CHART 3.—Composite hemoglobin and erythrocyte curves (average of 4 rats) showing the erythropoietic action of copper when added as the sole supplement to the raw milk diet of anemic rats.

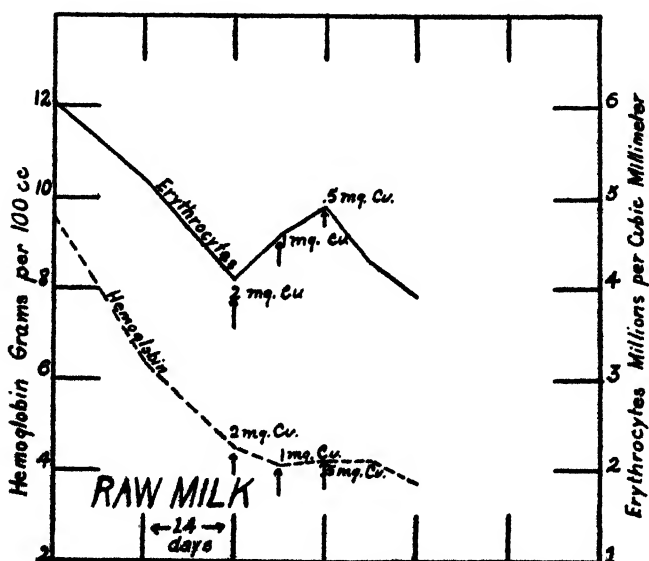


CHART 4.—Composite hemoglobin and erythrocyte curves (average of 4 rats) showing a relatively slight erythropoietic action when large doses of copper are added as the sole supplement to the raw milk diet of anemic animals.

When an attempt was made to duplicate these results by giving this larger dose of copper (2 mg.), it is interesting to note that the animals developed a severe diarrhea with an accompanying loss of weight. As it was evident that copper in this amount was toxic, the amount of this metal given daily was reduced after one week to 1 mg. daily. Since the diarrhea and loss of weight continued, the dose was decreased further at the end of the second week to 0.5 mg. of copper daily. Chart 4 shows that in these rats (4 animals) there was a temporary increase in the erythrocyte count with no rise in hemoglobin, thus confirming the observations made in the earlier experiments that copper has an erythropoietic action.

#### DISCUSSION

These observations that copper has a stimulating effect on erythropoiesis distinct from its "catalytic action" on hemoglobin formation do not receive as much support in the literature as would have been the case had hemoglobin determinations and erythrocyte counts both been made in the studies on nutritional anemia which have appeared in recent years. In such experimental work, where iron and copper have both been given as supplements to a milk diet, the results of the independent effect of copper on erythrocyte formation have been partially masked. However, Myers and Beard (2) have shown that, when small doses of copper (0.0025 to 0.01 mg.) are given daily to anemic rats on a milk-iron diet, the red cell count returns to normal in most instances at a considerably more rapid rate than does the hemoglobin. This would seem to indicate, though not definitely, that copper has a stimulating effect on erythropoiesis independent of its effect on hemoglobin formation.

More clear-cut results have been obtained by Schultze (4) in experiments where copper *without iron* has been added as a supplement to the milk diet of anemic rats. Under these conditions the lack of iron prevents hemoglobin formation, inasmuch as this element is a necessary constituent of the hemoglobin molecule. Thus, the possibility of confusing the effect of copper on erythropoiesis with its catalytic action on hemoglobin formation is avoided. Schultze has found, as we have done, that a rise in the erythrocyte count with no change in the hemoglobin follows the administration of the copper.

Another point of considerable interest revealed by the experimental data may be observed by the examination of Charts 1 and 2. It will be seen that the rate of fall of the hemoglobin with a diet of raw milk alone is much more rapid than when either evaporated milk or raw milk supple-



mented by copper has been fed. Evidently the presence of copper, both in the evaporated milk as well as when used as a supplement to raw milk, exerts its well known catalytic action on hemoglobin formation, even in the absence of a dietary source of iron, by bringing about a better utilization of tissue iron or of the iron liberated during the process of destruction of hemoglobin in bile formation.

On the basis of our experimental findings, supported by the work of others, we feel justified in drawing the conclusion that copper has two distinct rôles in blood formation: first, its well recognized catalytic action on hemoglobin formation, a point on which all workers in the field of nutritional anemia agree, and second, a stimulating effect on erythropoiesis.

#### SUMMARY

1. The feeding of evaporated milk to normal rats produces a fall in hemoglobin without the corresponding drop in the erythrocyte count that occurs when raw milk is the sole article of diet.

2. When copper without iron is fed as a supplement to raw milk, results similar to those with evaporated milk are obtained, indicating that the copper in the evaporated milk is responsible for the temporary maintenance of a high erythrocyte count.

3. When given as a supplement to the milk diet of anemic rats, copper ranging in amounts from 0.025 mg. to 0.5 mg. daily shows a definite erythropoietic action without any influence on hemoglobin formation.

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## Editorial Review

### THE POSSIBILITY OF THE CONVERSION OF FATTY ACIDS TO GLUCOSE IN THE ANIMAL BODY

**O**F ALL the possible interconversions of food nutrients in the animal body, the conversion of fats to sugar seems to have excited the most intense interest and to have divided scientific opinion the most sharply. The reasons for this unique characterization are not far to seek.

The conversion of protein to sugar in the diabetic animal and the conversion of sugar to fat in the heavily-fed farm animal are, and have been, readily demonstrated. The rapidity of conversion is so great as to be unmistakable. On the other hand, the conversion of sugar or fat to protein is out of the question, because the latter nutrient contains nitrogen and generally sulfur, two elements not found in sugar or fat. The fact that sugar and some of its probable metabolites, in conjunction with ammonium salts or some other simple nitrogenous compound, may be synthesized to one or two of the simpler amino acids is interesting, but falls far short of establishing any reasonable probability that from such precursors a complete protein molecule can be formed in the animal organism. The conversion of protein to fat in the higher animals is not easily proven, it is true, and at the present time, in the reviewer's opinion, it has not been rigorously demonstrated. Nevertheless, the evidence in favor of the conversion is weighty, and the similarities in the structure of some of the amino acids and of fatty acids, together with what is known of their metabolism, offer no serious obstacles to the belief that the conversion is possible. Furthermore, the transformation of protein to fat through sugar, though indirect and cumbersome, is thoroughly possible. Doubtless some day an ingenious experiment will be devised that is capable of establishing definitely that protein is convertible into fat, but there is little incentive (and unfortunately not much credit to be gained) merely to demonstrate what is almost universally believed to be true.

Contrast with these problems that of the conversion of fat to sugar, neglecting the glycerol constituent of the former. For almost fifty years it has engaged the interest of scientists, and yet so slow is the rate of conversion, if it exists at all, or so restricted the conditions permitting it, or so difficult is it<sub>L</sub> to devise an<sub>F</sub> effective experimental method, that the mass of

evidence is utterly confusing. As reviewed by some investigators, it is interpreted to mean that the conversion has been proven; other reviewers are as positive that the conversion is impossible, while the more moderate verdict of "not proven" is voiced by others. There are no *a priori* grounds for believing the conversion to be possible in the body. The difficulties inherent in the problem and the divergent views concerning its solution have both served to sustain interest in it, and today it is being as vigorously attacked as at any time since it was first conceived.

It will not be the purpose of this review to consider all of the experimental work concerned with the possible conversion of fatty acids to sugar. The many earlier reviews of this question, particularly the excellent critical discussion of Rapport (32) published in July, 1930, make such a course unnecessary. A general consideration of the different lines of approach will be attempted, but only those contributions appearing within the last two or three years, and in a few cases earlier studies intimately related to them, will be appraised in any detail. It appears unnecessary to discuss the glycerol constituent of fats in this connection, since the conversion of this compound to sugar in the diabetic organism, as well as in the phlorhizinized dog, has been firmly established.

The problem of the convertibility of fatty acids into sugar is related to other important physiological problems so intimately that its solution follows almost as a corollary from the solution of the latter. An example of such a related problem is that of the nature of the metabolic disturbance in diabetes. If this disturbance is essentially a partial or complete failure in the ability of the tissues to oxidize glucose, there are no implications concerning the ability of the body to form glucose from fatty acids. On the other hand, if the metabolic disorder is solely an over-production of glucose, the mechanism of glucose oxidation being unimpaired, then the low respiratory quotients in diabetes can be explained only on the assumption of a massive conversion of fat into sugar. The latter theory of diabetes has never been securely established, and in the last few years it has become less tenable than ever (9, 34, 35). In this country, at least, it is commonly believed that the "defective oxidation theory" of diabetes explains the observed facts much better than the "over-production theory."

Another illustration of the same point is the problem of the fuel of muscular contraction. Among a number of influential groups of physiologists in England, Germany, and Canada the theory that the muscles, and probably other tissues, can burn only sugar has been strongly advocated. Obviously if this theory is correct, fatty acids must be convertible into

sugar, because fats are demonstrable sources of muscular energy. MacLeod, a member of one of these groups, has called sugar the "fuel of life" and has written a book in defense of this thesis. The most direct evidence bearing upon the problem and responsible for this particular solution of it has been obtained, first, from chemical and thermodynamic studies of isolated muscle in various conditions of rest, activity, fatigue, and recovery, and, second, from observations on the respiratory exchange of intact animals during and following muscular activity.

The classical investigations of muscle chemistry and muscle energetics initiated in 1911 by Fletcher and Hopkins and continued brilliantly by Hill, Meyerhof and their collaborators, established many facts concerning muscular metabolism, facts that revolutionized current conceptions and opened up many avenues for further investigation. But in interpreting these facts in terms of concrete chemical reactions and in applying them to the muscle *in situ*, it now appears that they arrived at a solution of the problem which was much too simple. They assumed that the only chemical reactions occurring in isolated muscle that contributed appreciably to the energy transformations or the gaseous exchanges were the reactions involved in (or conditioned by) the anaerobic breaking down of glycogen to lactic acid in an initial phase, and the oxidative reconstruction of glycogen from lactic acid in a recovery phase at the expense of lactic acid, or possibly some other metabolite of glucose with an R.Q. of 1. Thus, the only source of muscular energy, according to this simple picture, is glucose, and the conversion of fatty acid to glucose becomes a necessary corollary. The further assumption that no other source of muscular energy is available to the muscle *in situ* appeared to be substantiated by a number of experiments on men during and after short periods of moderate muscular exertion. In such experiments, regardless of the value of the basal respiratory quotient, the quotient of the excess gaseous exchange approximated closely to 1. Hill has described, correlated and interpreted these different lines of evidence in a very readable article that appeared in *Science* for December 5, 1924.

Since then many important contributions have been made to our knowledge of muscle chemistry and muscle physiology, and the carbohydrate changes have assumed a secondary role. It appears that the anaerobic production of lactic acid furnishes, not the energy for muscular contraction as was formerly believed, but the energy to reverse the reaction of first importance, the anaerobic dissociation of phosphocreatine (phosphagen), thus recharging the muscle (30, 24). In this newer physiology, less cer-

tainty is expressed in the nature of the material that furnishes the energy for the resynthesis of glycogen in an isolated muscle, but even though this ultimately may prove to be carbohydrate in nature, there is no good reason to believe that the muscle *in situ* is so restricted in its choice of fuel. A tissue removed from the body has never been known, in so far as the author is aware, to acquire a function it formerly did not possess, but it obviously may lose a function that was dependent upon an intact supply of blood, lymph, and nerve impulses or upon the coöperation of other organs and tissues of the body. In fact, much evidence has accumulated to the effect that the respiratory quotient of the excess gaseous exchange produced by contraction of the muscles in the intact organism may assume a wide range of values. While this does not disprove the theory that sugar is the immediate fuel of the muscles, to which all other nutrients including fat must be converted, it does detract from the necessity of incorporating it into any complete explanation of muscular metabolism. Perhaps one reason why this theory has persisted so long and has obtained such a wide currency is that it is impossible to disprove it by any known method.

It is a fair judgment, therefore, that these modern investigations of muscular metabolism, although they have been so interpreted, have established no considerable probability for the theory that fatty acids are convertible into sugar in the animal body. The well-established fact, often cited in this connection, that muscular activity is performed more economically when the source of energy is sugar than when it is fat is consistent with the theory that the latter must be converted to the former for complete utilization, but it does not amount to competent evidence for it.

If the conversion of fatty acids to sugar occurs in the body, its effect would be to depress the respiratory quotient, since it would amount to a storage of oxygen. If R.Q.'s less than that for the oxidation of fat, i.e., 0.70 or thereabout, could be secured experimentally under conditions of a plethora of fatty acid molecules in the blood and intercellular fluids, then objective evidence of the conversion would be at hand, quite analogous in its significance to R.Q.'s greater than 1.00, obtained after large carbohydrate meals. It is true, the evidence would not be so clear cut, because a conversion of amino acids to sugar, the desaturation of fatty acids, and various intermediary oxidative reactions may have the same effect, and it must also be remembered that the failure to obtain such low quotients after fat feeding is no evidence against the possibility of the conversion, because the effects of a slow rate of conversion on the respiratory quotient may be entirely obscured by the simultaneous oxidation of a small amount of protein or of sugar.

In Murlin and Lusk's (31) studies of the specific dynamic effect of fat they fed 75 grams of the nutrient to dogs. In many of the experiments no significant depression of the basal R.Q. occurred and in no case was the hourly quotient depressed below 0.71. Gregg (16) working in Murlin's laboratory has determined the respiratory quotients of albino rats (weighing 150 to 200 grams) after the consumption of relatively large amounts of butterfat. Of all of the quotients obtained, only one, 0.634, may be regarded as possibly indicating a formation of sugar from fatty acids. Ten other quotients were less than 0.70, but they occurred during the early hours of fat absorption and according to Gregg, "are due without doubt to a transient acidosis and ketonuria." The average of all respiratory quotients obtained is 0.72, and the entire series can be explained without the necessity of assuming a formation of sugar from fatty acids. In this connection, it should be emphasized that an occasional atypical R.Q. considerably below 0.70 cannot be accorded full significance, because this quotient is ordinarily subject to a technical error of  $\pm 0.02$  and occasionally a much greater error may be committed. Observations of similar import on men consuming meals containing as high as 200 to 300 grams of fat have been reported by McClellan, Spencer, and Falk (28).

These results are typical of high-fat diets, and offer no support for the theory that fatty acids are convertible to sugar in the body. However, some observations from Murlin's laboratory indicate the possibility of forcing the R.Q. below 0.70 by feeding high-fat diets. Hawley, Johnson, and Murlin (21) reported such observations upon human subjects and Hawley and Murlin (22) similar observations upon a pig. Unfortunately these experiments have not as yet been reported in detail. It appears that the low non-protein R.Q.'s were corrected for any products of incomplete fat metabolism present in blood and urine. In the pig experiments it is stated that low quotients were obtained following the meal with a subsequent rise above the original level. This sequence of events suggests that gastric secretion with its withdrawal of chloride from the blood, and intestinal absorption with its restoration of chloride, may have produced these variations in R.Q. by causing a retention, followed by an expulsion of carbon dioxide from the body. This question has been discussed by McClellan, Spencer, and Falk (28). Murlin himself, in a private communication, frankly admits that "of course the evidence from low quotients must be checked by actual finding of the carbohydrate which is supposed to be formed."

Inexplicably, to the reviewer at least, indications of the conversion of

fatty acid to glucose based upon the respiratory quotient, have been obtained more frequently in the post-absorptive period than in the period following excessive fat feeding. During the post-absorptive period the metabolic activities are presumably almost entirely catabolic in character, while the glycogen stores, if they are not at their maximum for the dietary regime prevailing, have not been greatly depleted. When, under such conditions, respiratory quotients less than 0.70 are obtained, they are ordinarily dismissed as the result either of technical errors in gas analysis, or of a fasting ketosis, representing incomplete oxidation. Thus, the low quotients reported by Heinbecker (23) for Eskimos may be considered unreliable, because of the low oxygen percentages obtained for atmospheric air, indicative of an ineffective pyrogallol solution.

Recently, Lyon, Dunlop, and Stewart (27) have reported low total respiratory quotients for obese subjects in basal metabolism tests. These subjects were on reducing diets, containing 2000 calories or less per day. The quotients below 0.70 were particularly frequent for the subjects on a diet of 1000 calories, containing only small amounts of carbohydrates. For 26 determinations, calculations of the non-protein R. Q., the maximal sugar that could have been produced from protein, and the minimum amounts of sugar that must have been produced from fat are made. Throughout all tests, a routine examination of the urine for acetone was made. In a number of instances when acetone was present, the total excretion of ketones was determined quantitatively, but was never found to exceed 0.5 gram daily. The Douglas bag method was used, and the collection periods were of six minutes duration. Every point of possible error in technic seems to have been checked and the authors feel justified in concluding tentatively that "our experimental results afford evidence that in the undernourished obese subject, fat is converted into carbohydrate."

In an experiment in which the accuracy of the most significant results depends so intimately upon the accuracy of gas analyses, it is disturbing to find that the analyzers were tested daily, not with outdoor air of known and constant composition, but with laboratory air, which ordinarily cannot be so characterized. The calculations of the non-protein R.Q. and of the extent of the possible conversion of protein to sugar and of fat to sugar are carried out according to approved methods. While the point is not seriously involved in the validity of the main contention of Lyon, Dunlop, and Stewart, it may not be entirely impertinent to comment on the uncertain reliability of such methods of analyzing the data obtained from the respiratory metabolism and the excretion of urinary nitrogen. All such methods

are based upon a simplified and purely conventional picture of metabolism, in which there is no place for transformations of one nutrient into another within the range of respiratory quotients from 0.7 to 1. They involve a correction for protein metabolism based upon the urinary nitrogen as a criterion of the rate of protein oxidation, and the factors used for estimating the heat liberated and the gaseous exchange resulting from protein oxidation relate to one protein, generally, in this country at least, the protein of meat. The lag in the excretion of nitrogen liberated by deamination, as well as the fact that deamination of amino acids may occur without their complete oxidation, do not deter investigators from applying these methods to all conditions of metabolism and rarely elicit any misgivings anent the significance of the results obtained. The meat protein factors are used in interpreting the course of metabolism after food, whether meat, or some other protein differing widely from it in nitrogen content and in digestibility, is being consumed, or whether the subject is a dog, the animal on which the factors were obtained, or a steer. They are used in the analysis of basal metabolism data, during a period when a considerable part of the urinary nitrogen is probably a delayed excretion from the catabolism of the last meal, and when the endogenous end products, representing apparently a type of tissue catabolism not involving simply a protein oxidation, account for an increasing fraction of the urinary nitrogen. Fortunately the untenable assumptions upon which this method of indirect calorimetry is based may not appreciably impair the accuracy of the estimations of total heat produced within the range of respiratory quotients from 0.7 to 1, if these estimations have been adequately checked by direct calorimetric methods.<sup>1</sup> This circumstance is due both to the fact that the heat equivalent of a liter of oxygen is not greatly affected by the nature of the metabolic mixture, and to the fact that the heat equivalent of a liter of oxygen consumed in the oxidation of protein is not greatly different from that of oxygen consumed in the oxidation of a mixture of fat

<sup>1</sup> However, Adams and Poulton (*Proc. Physiol. Soc.*, 1932, 1P; *Jour. Physiol.* 77) have recently presented some disturbing evidence, from published calorimetric data, that the error in indirect calorimetry relating to total basal heat production is correlated with the respiratory quotient. They interpret this correlation to mean that, regardless of the observed R.Q., a constant proportion of carbohydrate and fat is being burned corresponding to an R.Q. of 0.785. The writer has made a study, similar to that of Adams and Poulton, of selected data from Lusk's numerous calorimetric experiments on dogs, the basis of selection being (1) that the directly determined heat production did not follow the administration of cold liquids, and (2) that the calculated heat production did not involve any but the ordinary assumptions. From a compilation of about 300 data, not however confined to basal periods, no appreciable correlation was detected between the percentage difference between observed and calculated heat and the total respiratory quotient.



and sugar corresponding to an R.Q. of 0.80, characteristic of protein oxidation. Hence, large errors in estimations of the extent of the participation of sugar, fat, and protein in the energy metabolism may coexist with considerable errors in the estimation of total heat production.

The contribution of Lyon, Dunlop, and Stewart is certainly suggestive of a conversion of fat to sugar, but the results of these experiments practically stand alone as regards the large proportion of basal respiratory quotients below 0.70. In the well-known studies of Means (29), some low quotients were obtained in individual tests, but they were not typical of any subject but one. Hagedorn, Holten, and Johansen (19), in basal metabolism studies on 16 normal and 30 obese subjects, obtained very few respiratory quotients less than 0.70, but they did note a distinct tendency for the quotients of the obese subjects to be lower than those of the normals, the difference averaging 0.05; all subjects were on the same high-carbohydrate diet. This difference is interpreted as the result of an abnormally increased transformation of carbohydrate to fat in the obese immediately after food, with a correspondingly lower respiratory quotient in the post-absorptive period.

The ability of the animal body to transform sugars, glycerol, lactic acid, and some of the amino acids into glycogen has been investigated by feeding these substances to experimental animals and then determining the concentration of glycogen in their livers after allowing a few hours for absorption. If the glycogen concentration is considerably higher in animals so fed than in control animals that received no food, the conclusion has been drawn that the substance fed has been converted to glycogen. While the evidence is not unassailable, since the participation of glycogen precursors from other tissues in the increase in liver glycogen cannot be ruled out, it may amount to practical certainty if the increases above the control level always occur and are always large. On feeding fats in such an experiment, no evidence has been obtained that fatty acids are convertible into glycogen. If animals are maintained for some time on a high-fat diet some glycogen formation is indicated, but no more than could be amply accounted for by the glycerol contained in the fats (16, 18). In a recent experiment, Greisheimer (18) fasted rats for 48 hours and then fed them different food materials during a 24 hour period; the amounts consumed were unfortunately not reported. The rats were then killed and the livers analyzed for glycogen. Four female rats fed lard showed an average concentration of 0.202 per cent of glycogen in the liver, as compared with the average of 9 control females of 0.135 per cent. Eight male rats gave an average of 0.715 per cent

glycogen against the control average (3 males) of 0.513 per cent. One male rat was forced fed with oleic acid, the amount given being again unstated. The liver glycogen in this case was 0.207 per cent. Contrasted with these negative results are the positive results obtained with glycerol, casein, sucrose, and the stock diet, which induced glycogen concentrations ranging from 1.81 to 7.82 per cent.

Experiments have also been carried out on the possible role of fatty acid metabolites in glycogen formation. Thus, Rosenthal (36) tested  $\beta$ -hydroxybutyric acid on fasting mice, but was unable to secure evidence of its conversion to glycogen in the liver. Eckstein (13) in similar experiments on fasting rats, investigated a series of lower fatty acids, including propionic, butyric, valeric, and caproic acids. Only propionic acid, an acid that has never been related to fat metabolism, gave evidence of conversion to glycogen in the liver. Butyric acid, a probable metabolite of naturally-occurring fatty acids, gave entirely negative results.

Another method of approaching the problem of the conversion of fats to sugar in the liver has been the perfusion experiment. Conflicting results have been reported in the literature, a situation not surprising in view of the fact that perfusion technic is extremely difficult and its refinement to anything approaching perfection is a very late accomplishment. At the present time it is realized that proper aeration of the excised liver by means of the perfusion fluid is extremely important, not only to the continued survival of the organ, but to the demonstration of its functional capacities. It is also evident that conclusions cannot be based merely upon the changes that occur in the composition of the perfusate. Particularly with reference to the problem under discussion, an examination of the changes that have occurred during the perfusion in the composition of the liver tissue itself is essential to a successful experiment.

In 1926, Burn and Marks (5) reported the results of a series of very carefully performed perfusion experiments on the livers of dogs and cats. In all experiments, increases in the total carbohydrate content of the perfusion system were observed, which could not be accounted for by decreases in lactic acid, or by increases in amino acid deamination and conversion of the non-nitrogenous residue into sugar. Although no attempt was made to determine whether the fat of the liver decreased during the experiments, the authors can conceive of no other source from which the sugar could have come.

The experiments of Burn and Marks have been criticized by Richardson (33, p. 97) because of the probable formation of lactic acid in the livers

under the anaerobic conditions that prevailed during the 20 to 30 minutes required for preparation for perfusion. He assumes that this lactic acid may have been the source of the sugar produced. However, the rate of disappearance of lactic acid was computed from initial analyses of samples of the perfusate taken 5 minutes after perfusion started. Presumably the lactic acid previously formed in the liver was present in this initial sample, so that its rate of disappearance was fully evaluated. It may be that the data of Burn and Marks are subject to considerable technical error,<sup>2</sup> and that their attempts to rule out non-fat precursors of the carbohydrate apparently produced were not successful, but taking the results as they have been interpreted, they constitute no evidence for the conversion of fatty acids to sugar. The gains in sugar reported varied from 0.3 to 1.5 grams, equivalent, in the two cases for which the necessary data are reported, to 0.3 and 0.6 per cent of the weight of the perfused liver, part of which may, according to the author's own data, have originated from lactic acid or amino acids. The residue may have been produced from glycerol liberated from the fats or phospholipids present in the liver tissue.

Jost (25) has more recently attempted to apply liver perfusion to the solution of the same problem. The perfusion experiments were carried out both with livers from dogs in a normal nutritive condition, and hence containing presumably a normal storage of glycogen, and with livers from fasting phlorhizinized dogs, which were rich in fat and poor in glycogen. The most striking results of these experiments were obtained after the addition of a phosphatid (either lecithin or cephalin) to the fluid being perfused through the fat-rich, but glycogen-poor, livers. On such addition, the reducing power of the perfusate was increased and the respiratory quotient of the liver determined from blood analyses was depressed. On the basis of these (and other) results it was concluded, first, that the catabolism of higher fatty acids takes place with the intermediate formation of phosphatids, and, second, that sugar may be formed in the liver from the fatty acids liberated from the phosphatids.

However, the significance of the gaseous exchange of such a preparation may well be questioned, because of the changes occurring in the CO<sub>2</sub> capacity of the perfusate and the incomplete oxidations of various descriptions that may be occurring, quite aside from the hypothetical conversion it is desired to demonstrate. The increase in reducing power of the perfusate,

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<sup>2</sup> Perhaps one indication of technical error is their failure to find any appreciable amounts of glycogen in the livers of fat-fed animals. This is quite a unique finding, contrasting with the recent findings of Gregg (17) and of Kapeller-Adler and Rubinstein (26).

if this may be taken to represent sugar only, may have been the result of a glycogenolysis induced by the lecithin (or cephalin) added to the perfusion fluid. The fact that little glycogen was found in the livers of the phlorhizinized dogs, does not mean that more might not have been present, since the determination of small amounts of glycogen by the Pflüger method yields minimal results (14). The effect of lecithin upon the carbohydrate metabolism of the intact animal seems to be a stimulation of glycogenolysis according to Cruto (10, 11). Its specific effects upon the liver of normal and depancreatized animals is being studied by Best and his associates (2, 3). The effects on the content of the liver in fat, phospholipins, and glycogen are suggestive of a conversion of fat to sugar, but a final decision on this question awaits the results of further study.

Gregg, working in Murlin's laboratory, has contributed the latest report (17) on liver perfusion, using the liver of fat-fed cats and dogs. In seventeen such experiments he observed the following in the perfusion system: a decrease in total carbohydrate, a decrease in total lipids, a steady production of urea, ammonia, and sugar, a smaller production of lactic acid and nonfermentable reducing substances, a marked constancy in the fatty acids of the perfusate, and a decided irregularity in the distribution of carbohydrates in livers containing them in small concentrations. No support for the theory of gluconeogenesis from fatty acids was obtained, and Gregg expresses the following significant judgment of the value of the perfusion method in the solution of this problem:

"Due to the changes necessarily inherent in such an unphysiological system, namely, lactic acid formation, glycogenolysis, metabolic use of sugar by the liver, edema of the perfused organ associated with marked unevenness of the distribution of sugars, and finally, the ineradicable possibility that sugar may always be coming from glycerol, it is well nigh impossible to use such a system to demonstrate the neogenesis of carbohydrate from fatty acids on a quantitative basis."

The diabetic state has always been considered a fruitful one for the demonstration of the conversion of nutrients or metabolites into sugar. Furthermore, it may readily be induced experimentally by pancreatectomy. The poisoning of animals with phlorhizin also induces a condition similar to pancreatic diabetes in that gluconeogenesis is greatly stimulated, while sugar oxidation is correspondingly inhibited.

The post-absorptive respiratory quotient in either clinical or experimental diabetes is generally low, and not infrequently below the R.Q. for fat. This, however, is not indicative of the conversion of fatty acids to

sugar, since the metabolic derangements in diabetes modify profoundly the significance of the quotient, particularly the incomplete oxidations of fatty acids and amino acids and the accumulation of acidic metabolites in the body fluids. Although the calculations designed to gauge the effects of these derangements of metabolism upon the respiratory quotient are not particularly convincing, it does appear that their net effect will be to lower the oxidative respiratory quotient. Hence, it does not appear to be necessary in this case to enlist the services of a hypothetical conversion of fatty acid to sugar.

Phlorhizined dogs will readily produce sugar from proteins and many of the amino acids, and the formed sugar is excreted so promptly and so invariably that it has been possible to compute the yield of sugar from different proteins and even the number of carbon atoms in any glucogenetic amino acid that is carried over into sugar. But the ingestion of fat by phlorhizined dogs has elicited no such response in the large majority of experiments of this type, and the result is the same when fats are fed to diabetics. In those few studies in which the production of extra sugar has been claimed, the amount is so small and the dextrose to nitrogen ratio in the urine so variable that the evidence is of doubtful validity. For example, in Geelmuyden's experiments (15) the effect of fat on the sugar excretion of phlorhizined rabbits was tested with 5 animals. In three experiments the nitrogen in the urine as well as the urinary sugar was determined. In only two of these cases was any disturbance in the D:N ratio produced by fat feeding, and the slight disturbance noted occurred, not on the day of fat-feeding, but on the second and third day following. Obviously, in the two experiments in which the urinary nitrogen was not determined, any disturbances noted in the excretion of urinary sugar cannot be profitably discussed.

Probable metabolites of the higher fatty acids, such as butyric and acetic acids, are also without effect on the D:N ratio of phlorhizined animals. Geelmuyden's experiments (15) are again quite negative in their significance, although interpreted in a positive fashion by the author, who consistently refused to give to an increased nitrogen excretion in such experiments its customary and logical significance. Deuel and Milhorat (12), in much better controlled experiments on phlorhizined dogs, observed no indications of a conversion of acetic acid to sugar.

Soskin (37) attempted to induce a conversion of fatty acids to sugar in depancreatized dogs by feeding them fat (either olive oil, cottonseed oil, butter, or "intarvin") with lipase, or lecithin. The test substances were

given to the dogs on the fifth day after the withdrawal of food and insulin. In 10 of 13 experiments, Soskin was unable to secure evidence of the conversion of fatty acids to sugar, but in 3 experiments, the same type of calculation indicated the excretion of amounts of sugar (5.28, 4.85, and 6.91 grams) that were not traced to glycerol or catabolized protein. In all of these cases the dogs died on the day following the experimental feeding, which consisted, in one case of 40 grams of lecithin (15 grams given subcutaneously), in the second case, of 50 grams of olive oil and 50 cc. of castor bean suspension, and in the third case, of 30 grams of lecithin. The 10 negative experiments, in all of which the dogs survived, merely illustrate in the opinion of Soskin "the physiological limitations of the method."

In the 3 experiments interpreted in a positive fashion the R.Q.'s were not so low as to suggest a conversion of fatty acid to sugar, being 0.69, 0.71, and 0.71, and the possibility that glycogen stores might have been the source of the extra sugar as calculated is dismissed as "unlikely. There is at least no evidence that the administration of a food material like a neutral fat can cause a displacement of glucose from the tissue stores and it can be seen from the tables that the control animals in the present experiments showed no such displacement." The control animals were given mineral oil. No critical comment on these experiments is called for. Their mere description indicates the intangible character of the evidence adduced.

Much controversial material has been published concerning the significance of the dextrose to nitrogen ratio in clinical and experimental diabetes. This controversy has revolved mainly around the contention that, in pancreatic diabetes, the ratio assumes a fairly constant value approximating 2.8 to 1, while in phlorhizin diabetes it approximates 3.6 to 1, and that these ratios measure the extent of the conversion of protein to sugar under the respective conditions. Since the discovery of insulin and its use in preserving indefinitely the lives of depancreatized animals, it has been possible to study the dextrose to nitrogen ratio under better controlled conditions and on dogs in a much better physical state. Variations have been observed in the ratio which have been interpreted as evidence that, after the glycogen stores are depleted, other nutrients than protein are contributing to the urinary sugar. Chaikoff (6) believes that there is a relation between the ratio and the condition of the animal such that fat animals tend to exhibit a high ratio and thin animals a low one. Arguing that D:N ratios greater than 3.65 constitute "indisputable evidence that sugar is derived from fatty acid," when proper allowance is made for the possible

formation of sugar from glycogen stores and glycerol, Chaikoff concludes that the data for one of his four dogs (Dog C) support the view "that glucose is derived from fatty acid in the diabetic organism."

The argument is illogical, since it employs a D:N ratio the significance of which rests upon the assumption that fatty acids do not contribute to sugar formation, to prove that fatty acids are in fact so convertible. Furthermore, in this and all other experiments of this type it is impossible to rule out all participation of glycogen stores in the formation of urinary sugar on the basis of estimates of the probable glycogen content of the animal from averages of analyses on other dogs. This is true, first, because of the variations exhibited among dogs with respect to their glycogen stores: a small group of analyses affords no sure basis for setting an upper limit to the glycogen stores compatible with a given condition. In particular, as Rapport (32) has aptly pointed out, fat (well-nourished) dogs, which showed the suspiciously high ratios, may be expected to contain exceptionally large stores of glycogen as well as of fat, all the more so if Wertheimer (38) is correct in his finding that adipose tissue itself may contain as much as 1 per cent of glycogen.

A second objection to such calculations rests on the inaccuracy of the glycogen determination itself (14), but particularly upon the glycogenolysis that invariably occurs previous to analysis of muscle and liver. Cori (8, p. 162, 174, 175) has clearly described and emphasized these sources of error, particularly the glycogenolysis following death: "When an animal is killed by stunning, bleeding, decapitation, administration of ether or chloroform, the resulting asphyxia as well as strong nervous discharges, both acting at a temperature of 37°C., cause a rapid breakdown of glycogen. A muscle which is removed under these conditions even if it be frozen at once shows a lactic acid content which is considerably above the normal resting value and also considerably higher than the lactic acid content of the blood." As an indication of the order of magnitude of the error thus induced, Cori cites a value of  $0.37 \pm .03$  per cent of glycogen in the gastrocnemii of fasted rats after killing, and a value of  $0.59 \pm .04$  per cent when the muscles were removed from the living animal under amytal anesthesia. Anderson and MacLeod (1) have more recently studied this post mortem glycogenolysis, particularly with reference to the death of the muscles themselves, and have stated that "no method was found in which the muscle could be killed without causing stimulation of the reaction leading to the disappearance of glycogen. A post mortem increase of lactic acid, usually out of proportion to any glycogen decrease, was always found under

these conditions." It must be concluded that all glycogen analyses of animal tissues possess a minimal significance, and that, under the conditions that usually obtained in published experiments, they may be very greatly in error.

In 1926 Wertheimer (39), convinced that fats are convertible to sugar, tested the possible catalyzing effect of insulin on the reaction. He compared the reaction to insulin, first, of dogs whose bodies had been depleted of glycogen in a preliminary phlorhizin period and whose livers had accumulated an abundance of fat, and, second, of dogs that had been subjected to fasting only or had had their normal ration up to the time of insulin administration. He found that the phlorhizinized dogs were less sensitive to insulin shock than the unphlorhizinized dogs, many of which died shortly after the insulin injection. Their blood sugar returned to normal levels quicker (or even attained hyperglucemic levels); their livers lost fat, but gained glycogen. Hence, the conclusion was drawn that fats had been converted to sugar, which exerted its well known antidotal effect on insulin shock. The possibility that protein was the precursor of any sugar or glycogen formed is dismissed with the statement that protein catabolism was unchanged, though an increase in protein catabolism is characteristic of effective phlorhizinization of dogs. Wertheimer's interpretation of his experiment ascribes to insulin a function that no other experiments concerned with the physiological effects of the hormone have even suggested, insofar as the reviewer is aware, and the experiment, viewed in the light of this interpretation, may almost be said to "prove too much" in implying that, in the insulinized animal, sugar is more readily produced from fat than from glycogen.

The conception of Wertheimer has been submitted to a much more thorough test by Hawley (20), who used the same dogs, first in a fasted and then in a phlorhizinized condition, for insulin treatment. She also determined the respiratory exchange of the dogs during the first 6 to 8 or 9 hours after administration of insulin. In these experiments, the animal after phlorhizinization was in no case more resistant to insulin than after fasting, and the respiratory quotients in no case suggest a conversion of fat to sugar. It is true that a negative experiment of this type cannot, by that fact alone, invalidate an experiment possessing an opposite significance, but its results may be given more weight if they are based upon a more comprehensive set of data involving less biological error, as these were, and if the interpretation that they support more readily dovetails in to the established facts and generally accepted theories of metabolism.



The primary effect of epinephrine on carbohydrate metabolism is an acceleration of glycogenolysis in all cells. When epinephrine is injected into a depancreatized dog an increase in the D:N ratio may follow, the extra glucose originating from the glycogen stores of the tissues. Repeated injections generally have failed of this effect. In 1928, Chaikoff and Weber (7) attempted to account quantitatively for the sugar excreted in the urine of depancreatized dogs in response to epinephrine injections. On the third and fourth days after the last meal and the last injection of insulin, depancreatized dogs were given repeated injections of epinephrine. The extra sugar excreted in the urine was compared with the sugar that might have been formed from liver glycogen, protein, or glycerol, and in 8 of 9 tests, the sugar formed exceeded the given sources of sugar. Although the authors believed that muscle glycogen could not, on the basis of published work, have contributed to the urinary sugar, its possible effect was nevertheless estimated. With this added source of sugar, there were still three tests in which the extra urinary sugar exceeded the sum of the quantities of sugar from all recognized sources. Although the sugar and glycogen contents of the animals were not determined either before or after the experiments, being estimated on the basis of previous analyses on dogs in similar condition, Chaikoff and Weber concluded that the extra sugar, in some of the experiments, must have been derived in part from fatty acids.

Rapport (32) has criticized the experiments of Chaikoff and Weber on the following points: the glycogen stores were not stabilized at the time of epinephrine injection, since the D:N ratios had not reached a level; the impossibility of making any accurate estimate of the "extra sugar" excreted because of the extreme variability of the dextrose to nitrogen ratios in the urine; the assumption that muscle glycogen could not have contributed to the extra sugar; the respiratory quotients are not in harmony with the assumed conversion of fat to sugar. To these criticisms may be added the probability of considerable error in the assumed glycogen contents of both muscle and liver. The question of the possibility of muscle glycogen contributing to epinephrine glycosuria was answered very definitely in the affirmative by Bollman, Mann, and Wilhelmj (4), who repeated the experiments of Chaikoff and Weber, but analyzed samples of muscle, blood and liver of each dog, four being used, both before and after the period of epinephrine injection, throughout which time the urine was obtained by catheterization. Their calculations show, not only that the muscle glycogen stores are depleted by epinephrine, but that the sugar thus formed amply accounts for the sugar in the urine in excess of that

produced from protein; the glucose and glycogen of the liver and the glucose of the muscle were not appreciably changed. It may be noted that though all glycogen analyses made on muscle or liver may be much lower than the true values, for reasons already given, the differences in the values obtained before and after the epinephrine injections may have been substantially correct, if the glycogenolysis initiated by the removal of tissue samples may be assumed to proceed to a like equilibrium determined by the concentration of lactic acid capable of inhibiting further change. These experiments of Bollman, Mann, and Wilhelmj may be considered to dispose completely of Chaikoff and Weber's interpretation of their own experimental data.

This review of recent contributions to the problem of the possible conversion of fatty acids to sugar demonstrates the elusiveness of the definite solution. The methods of experimental attack are indirect, perhaps necessarily so, and the logic involved in the interpretation of the results so often involves debatable propositions, or such naïve expectations as that in fat animals the catabolism of fat and hence the rate of its conversion to sugar, are more intense than in thin animals, or that a glycogenolytic agent such as epinephrine should catalyze reactions leading to the formation of more sugar, or possibly more glycogen, to feed the glycogenolysis mechanism. Without exception the evidence for the conversion is not directly associated with fat, but, by a process of more or less successful elimination, with a substance that cannot conceivably (to the investigator himself) be anything else but fat. Granting the validity of the evidence, such an association may be tantamount to a demonstration, but on the other hand it may be merely a result of an incomplete state of knowledge of animal metabolism, or even a revelation of a mental bias, or of a lack of imagination in the investigator. The significance of most of the information yielded by these indirect attacks on the problem must be discounted, if it is not entirely invalidated, by later investigations that reveal unsuspected technical errors, or furnish alternative explanations of the data, or prove that the data are, for some unexplained reason, quite atypical of the experimental conditions.

The sum total of the evidence for the possibility of a conversion of fatty acids to sugar seems to be merely suggestive, certainly far from conclusive. On the other hand, there is no justification for a catagoric denial that the conversion is possible. The verdict of "not proven" is the most logical one to return.

In concluding this discussion it may not be presumptuous to suggest a

new line of attack. The mammary gland is concerned with the continuous secretion of sugar, and possibly with its continuous manufacture. The milk formed by the gland is passed to the exterior where it may be weighed and analyzed. If this secretion can be maintained on a high-fat diet in any species of mammal, without destruction of body protein and after depletion of body glycogen, at such a rate that the non-fat constituents of the diet could not support it, a high probability could be established that dietary fat was the source of the lactose formed. Negative results would of course possess little significance, since fat may be a specific inhibitor of lactation.

H. H. M.

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GROWTH OF RATS FED HIGH PROTEIN RATIONS  
SUPPLEMENTED BY DIFFERENT AMOUNTS  
AND COMBINATIONS OF VITAMINS  
B (B<sub>1</sub>), G (B<sub>2</sub>), AND B COMPLEX\*†

By

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WHEREAS it is generally conceded that a ration rich in protein is adequate for carnivora, for many years investigators have expressed their conviction that omnivora and herbivora suffer in some manner from such a regimen. Besides attributing to high protein dietaries a deleterious effect upon renal and vascular tissue, some workers claim that restricting an animal to such food during the period of rapid development results in a retardation of the rate of growth. Reports, however, are conflicting. A survey of the literature on the subject indicates that both normal and subnormal rates of growth are recorded for rats subsisting on rations containing 45 to 95 per cent of casein.

An opinion has also been expressed that a diet having a high protein constituent increases the animal's need for B vitamins (1, 2). Sherman and Gloy (3) investigated this subject by employing rations containing 12 to 54 per cent of casein supplemented with orange juice as the source of vitamin B complex. Within the limits of their experiment, these authors found no basis for the belief that the rat's need for vitamin B complex is influenced by the proportion of protein in the food consumed.

A retarding effect upon the rate of growth of rats as the protein constituent of the ration increased beyond 14 per cent, has been reported by

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Slonaker (4). In the mixture of foods which he used, vitamins of the B group were contributed chiefly by 1.9 per cent of yeast and 2.8 per cent of wheat germ; without doubt these vitamins were contained also in other dietary constituents such as whole wheat, whole corn, and commercial skimmed milk powder. To increase the protein content above the lowest level (10.3 per cent) which this ration supplied, Slonaker states that meat scrap was added to it in such amounts as to increase its protein component to 14.2, 18.2, 22.2, and 26.3 per cent respectively. As no mention is made of the addition of yeast or of some other potent carrier of the B vitamins to these four diets, it is assumed that such adjuvants as were contained in the unmodified food must have been diluted progressively as meat scrap was added in increasing amounts.

The best rate of growth is reported for rats maintained on the ration which contained 14.2 per cent of protein. In the diet which furnished the highest level of protein used in Slonaker's investigation, the yeast and wheat germ components evidently had been reduced to 1.2 and 1.9 per cent respectively. He states that half of the females restricted to this latter dietary were sterile. Such an incidence of sterility as well as a retarded rate of growth due to the ingestion of protein in this or even higher quantities, are findings which have not been confirmed by other laboratories. Possibly incipient dietary deficiencies were responsible for the retarded growth and the abnormalities which Slonaker observed.

The data here presented were collected during the progress of another investigation (5) for which most of the rats had been subjected to a right unilateral nephrectomy. That this procedure, *per se*, does not interfere with the growth of the rat has been shown by Smith and Jones (6). Moreover, each experimental nephrectomized rat restricted to the food containing either 50 or 90 per cent of casein, is compared with a nephrectomized rat maintained on a control diet. In addition to this, other groups with both kidneys intact, have been fed both the high-protein and the control diets.

#### EXPERIMENTAL PROCEDURE

Female albino rats were used in this investigation which covered a period of 56 days. The fourteen groups which were nephrectomized were operated upon at the beginning of the experiment when 30 days of age. Four groups of intact animals also were used.

Diets containing 18, 50 and 90 per cent of casein (Table I) were fed *ad libitum* and vitamin-bearing adjuvants were supplied apart from the ration six times a week. Each rat received a daily allowance of four drops of

TABLE I  
COMPOSITION OF EXPERIMENTAL DIETS

Rat Groups	A	P	Z
	%	%	%
Casein*	18	50	90
Dextrin†	76	44	4
Agar‡	2	2	2
Salt Mixture§	4	4	4

\* Casein no. 453—Casein Mfg. Co., Bainbridge, N. Y.

† Commercial Dextrin, white. Eimer and Amend, N. Y.

‡ Agar U.S.P. coarse powder—Merck & Co., New York.

§ Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 919, 37, 557

cod liver oil (116 mgm.) and two drops of wheat germ oil<sup>1</sup> (43 to 45 mgm.).

All the groups of rats which ate a ration having 18 per cent of protein are designated by the letter A; similarly, P indicates those groups restricted

TABLE II  
DIETARY SIGNIFICANCE OF THE NAMES OF THE GROUPS OF RATS

Group names designating casein constituent of diet			Amounts and combinations of vitamins B, G and B complex represented by numbers 1 to 7 in the group names
% 18	% 50	% 90	
A1	P1	Z1	0.4 gm. of yeast
A2	P2	Z2	0.8 gm. of yeast
A3	P3	Z3	1.6 gm. of yeast
A4		Z4	1.6 gm. of yeast plus 3 drops (88.5 mgms.) of tikitiki extract daily.
A5		Z5	1.6 gm. of yeast plus autoclaved yeast mixed into the ration at a 7 per cent level until consumption reached 10 gms. of food daily, then the quantity was reduced to a 5 per cent level.
A6		Z6	0.8 gm. of autoclaved yeast plus tikitiki extract. 1 drop (27.5 mgms.) was given for the first 22 days, the amount was then increased to 9 drops weekly, then to 2 drops daily and finally to 3 drops daily.
A7		Z7	5 drops of tikitiki extract (147.5 mgms.) were fed during the first 46 days, the drops were then increased to 6 and finally to 12 daily. On the 28th day of the experiment and thereafter, 0.4 gm. of autoclaved yeast was given daily.

<sup>1</sup> Kindly furnished through the courtesy of Mr. M. W. Tapley of E. R. Squibb & Sons, Brooklyn, New York. Wheat germ oil no. 665 AH.



to the food containing 50 and Z, 90 per cent of casein. The letter K preceding A or Z indicates that all rats in groups so designated, were animals having two kidneys.

The amounts and combinations of vitamins B ( $B_1$ ), G ( $B_2$ ), and B complex supplied to each rat are indicated in the group names by numbers (Table II). These adjuvants were furnished by dried yeast,<sup>2</sup> tikitiki extract,<sup>3</sup> and autoclaved yeast, and were fed six times a week apart from the food mixture, except as specified to the contrary.

### DISCUSSION OF RESULTS

The level of dietary protein is only one of many factors (7) which may stimulate or depress the rate of growth of rats. In addition there are doubtless uncontrollable conditions which influence growth; even in paired feeding experiments, Mitchell and Carman (8) have observed that the gain in weight of rats is subject to variation because of the influence of what these authors term "unknown factors." Nevertheless, we feel that the importance of such unknowns is likely to be greatly minimized in the interpretation of our data, because of the numbers of rats represented in each group (Table III).

The mean gains in weight shown by groups of nephrectomized rats are not significantly different from the gains of intact groups restricted to the same ration (Table IV), even though the food calories consumed by 50 per cent of the groups which had two kidneys, are significantly higher than the caloric consumption of corresponding A and Z groups. This condition indicates that the nephrectomized rats have utilized food for growth better than have the rats possessing two kidneys. It is striking that this similarity in growth between the two groups should be maintained in the face of an excessive amount of dietary protein.

An adequate food mixture containing 90 per cent of casein and supplemented with 0.4 gm. of yeast produces inferior growth in both nephrectomized (Z1) and intact (KZ1) rats when they are compared with their respective controls (A1 and KA1) (Table V), as indicated by the significance ratios. However, there is no significant difference between the gains in weight of the Z1 and KZ1 groups (Table IV).

When the yeast allowance was increased four times, the intact rats (KZ3) grew as well as their controls (KA3) but the nephrectomized rats (Z3) made significantly inferior gains in weight in comparison with their

<sup>2</sup> The yeast was obtained from the Northwestern Yeast Company, Chicago.

<sup>3</sup> Kindly furnished through the courtesy of Dr. Brown, Philippine Bureau of Science, Manila, P. I.

TABLE III

MEAN GAINS IN WEIGHT AND FOOD INGESTED BY GROUPS OF RATS MAINTAINED FOR 56 DAYS ON RATIONS CONTAINING 18, 50, AND 90 PER CENT OF CASEIN SUPPLEMENTED BY DIFFERENT AMOUNTS OF VITAMINS B, G, AND B COMPLEX

Group	Mean gain in weight	Probable error	Significance ratio	Food calories ingested*				Number of rats
				Protein	Total	Probable error	Significance ratio	
A2	gms. 143	±3.55		454	2483	±46		14
A4	129	±4.85	2.33	527	2678	±81	2.1	12
A3	129	±2.97	3.02	480	2414	±41	1.1	20
A1	128	±2.34	3.53	399	2308	±51	2.5	14
A5	112	±3.25	6.44	496	2358	±45	1.9	13
A7	108	±2.27	8.31	374	2293	±28	3.5	12
A6	83	±3.95	11.30	350	1888	±57	8.1	12
P2	135	±3.43		939	2222	±12		9
P3	133	±4.52	0.06	998	2338	±28	3.7	9
P1	128	±3.39	0.29	878	2091	±19	5.7	9
Z4	118	±5.87		1672	2175	±52		12
Z5	114	±3.45	0.58	1690	2238	±54	0.8	12
Z2	113	±3.72	0.72	1489	2033	±56	1.9	14
Z1	100	±2.59	2.80	1418	1910	±56	3.5	14
Z3	97	±2.96	3.19	1488	2086	±60	1.1	14
Z7	94	±2.52	3.75	1519	1865	±41	4.7	12
Z6	67	±2.58	7.95	1187	1520	±41	9.9	12
KA1	128	±4.04		466	2724	±52		11
KA3	119	±3.86	1.58	508	2509	±54	2.9	13
KZ3	110	±4.05	3.09	1686	2197	±21	9.4	13
KZ1	107	±2.69	4.30	1651	2056	±40	10.2	12

\* Protein and total calories include those derived from yeast.

$$PE \text{ (Probable Error)} = \frac{\sigma \times 0.6745}{n}$$

$$\sigma \text{ (Standard Deviation)} = \sqrt{\frac{\sum d^2}{n-1}}$$

$\sum d^2$  = sum of the deviations from the mean, squared.

n = number of observations

SR (Significance Ratio) =  $\frac{D}{PEd}$  When the value is 3 or over, the difference between the 2 means is considered significant.

D = difference between two means.

$$PEd \text{ (Probable Error of the Difference)} = \sqrt{(PE \text{ mean } 1)^2 + (PE \text{ mean } 2)^2}$$

TABLE IV

A COMPARISON OF THE MEAN GAINS IN WEIGHT AND FOOD INTAKE OF GROUPS OF NEPHRECTOMIZED AND INTACT RATS OVER A PERIOD OF 56 DAYS

Group	Mean gain in weight	Significance ratio	Total food eaten*	
			Calories	Significance ratio
A1 KA1	gms. 128 128	0.00	2308 2724	5.7
A3 KA3	129 119	2.05	2414 2509	1.4
Z1 KZ1	100 107	1.88	1910 2056	2.1
Z3 KZ3	97 110	2.59	2086 2197	6.7
P1 KA1 KZ1	128 128 107	0.00 4.81	2091 2724 2056	11.4 0.7
P3 KA3 KZ3	133 119 110	2.35 3.78	2338 2509 2197	2.8 4.0
Z1 KA1	100 128	5.83	1910 2724	10.7
Z3 KA3	97 119	4.52	2086 2509	5.2

\* Total calories include those derived from yeast.

controls (A3) (Table V). Yet a comparison of Z3 with KZ3 (Table IV), shows no significant difference between their gains in weight. Rats with two kidneys seem, therefore, to tolerate such an excessive level of dietary protein no better than rats with only one kidney. Possibly the shorter experimental period which we have used may account for the differences of these results from those of Smith and Jones (6), who found a retarded rate of growth in nephrectomized rats if they were confined to a ration containing more than a moderate amount of the protein constituent.

Within the limits of our experiment, it appears that the growth of

nephrectomized rats is not hindered by a 50 per cent level of casein in the diet (Table IV). The mean gain in weight of the P1 group equals that of the KA1 group and exceeds that of the KZ1 group. The same statement applies to the P3 group when it is compared with intact groups of rats fed the control or the high protein rations, each supplemented with the same yeast allowance as P3 received.

TABLE V

COMPARISON OF THE GAINS IN BODY WEIGHT AND OF FOOD CONSUMED BY RATS WHOSE RATIONS CONTAINED DIFFERENT LEVELS OF PROTEIN

Group	Mean gain in weight	Significance ratio	Food calories ingested*	
			Total	Significance ratio
A4 Z4	gms. 129 118	1.44	2678 2175	5.2
A5 Z5	112 114	0.42	2358 2238	2.0
A2 Z2 P2	143 113 135	5.83 1.64	2483 2033 2222	6.2 5.4
A1 Z1 P1	128 100 128	8.02 0.00	2308 1910 2091	5.3 3.9
KA1 KZ1	128 107	4.33	2724 2056	10.2
A3 Z3 P3	129 97 133	7.64 0.74	2414 2086 2338	4.5 1.5
KA3 KZ3	119 110	1.61	2509 2197	5.4
A7 Z7	108 94	4.13	2293 1865	8.6
A6 Z6	83 67	3.39	1888 1520	5.2

\* Total calories include those derived from yeast.

A revived interest in growth has been stimulated by some recent papers (9, 12). It is timely, therefore, to make a comparison of the ability of rats to grow when they are limited to dietaries differing either in the quantity of casein, or in the amount or combinations of the vitamin B ( $B_1$ ), G ( $B_2$ ), or B complex supplements.

The mean gains in weight of all groups appear in Table III, while in Table V each experimental group is compared with its respective A or KA control. Among all the 21 groups represented, the most rapid growth was achieved by the A2 group of rats. A comparison of this group with the P groups and also with the Z group which gained the most weight, shows that over 66 per cent of the rats fed a ration containing 50 per cent of casein (Table VI) grew as well as those eating a diet in which the casein

TABLE VI

COMPARISON OF THE GAINS IN WEIGHT OF THE P GROUPS, ALSO OF THE MOST RAPID-GROWING Z GROUP, WITH THE A GROUP OF RATS WHICH GAINED THE MOST IN BODY WEIGHT

Group	Mean gain in weight	Significance ratio
	gms.	
A2	143	
P2	135	1.62
P3	133	1.74
P1	128	3.04
Z4	118	3.64

constituent was 18 per cent. Our results are thus in agreement with those of Sherman and Gloy (3) and with Smith and Moïse (10) in experiments in which rations containing 60 per cent of casein were fed to young rats. The most rapidly growing Z group of rats, however, made gains slightly inferior to those of the animals in the A2 group. This comparison is irrespective of differences in allowances of vitamin B, G, and B complex supplements.

The groups Z1, Z2, and Z3, which were given a ration identical in vitamin supplements with those consumed by the three P groups, made mean gains in weight inferior to those of the animals in the P groups (Table VII) and also to those of their own controls (Table V) A1, A2, and A3 respectively. Of the remaining Z groups whose vitamin adjuvants differed from those given to the rats in the P groups, Z4 and Z5 have grown as well as their

controls, but Z6 and Z7 made gains in weight which are significantly poorer than their own controls.

Thus, though the ration containing 50 per cent of casein permits an animal to grow as well as its controls whether they have two kidneys (Table IV) or only one (Table V), a ration having 90 per cent of this protein produces growth equivalent to that of controls in only 28 per cent of the nephrectomized groups (2 out of 7 cases, Table V) and in 50 per cent of those whose rats had two kidneys (1 out of 2 cases, Table V). The best growth reported by Osborne and Mendel (11) was attained by the use of a ration containing 35 per cent of casein, and the most rapid rate of

TABLE VII

COMPARISON OF THE GAINS IN WEIGHT OF P AND Z GROUPS OF RATS WHICH CONSUMED THE SAME AMOUNTS OF YEAST SUPPLEMENTS

Group	Mean gain in weight	Significance ratio
P1 Z1	gms. 128 100	6.52
P2 Z2	135 113	4.32
P3 Z3	133 97	5.66

growth yet reported was secured by Anderson and Smith (12), who fed to their rats a stock diet which furnished 33 per cent of protein from one source and 35 per cent from another food mixture, both of which were freely available to the rats at all times.

From the evidence available at present, the optimum level of dietary protein for rats lies between 18 and 50 per cent and probably between 30 and 40. Our results and those quoted above do not confirm Slonaker's findings.

The supplements which promoted the best growth in rats restricted to the control diet are 0.8 gm. of yeast, or 1.6 gm. of yeast fortified by tikitiki extract (Table III). Those which stimulated growth best when the rats were confined to the high-protein ration, are tikitiki extract or autoclaved yeast when either one was added to the highest level of yeast (1.6 gm.). In comparing the Z groups among themselves (Table III), 0.8 gm. of yeast

is just as effective as 1.6 gm. supplemented with either autoclaved yeast or tikitiki extract, but when comparing the mean gain in weight of Z2 with its control, A2 (Table V), 0.8 gm. of yeast is less effective than the other two supplements, for Z4 and Z5 are the only ones among all the seven Z groups which made as satisfactory gains as their respective controls.

The need of rats maintained on high-protein regimens for additional B or G vitamins, besides that furnished by yeast, has been emphasized by Hassan and Drummond (1). On the other hand, a comparison of the gains in weight of groups of rats which received *yeast alone* as the source of the B vitamins (Table VIII) does not support the thesis of Reader and Drummond (2), for increasing the supply of yeast to 1.6 gm. did not improve the rate of growth of any of the groups which we have studied.

As yeast is still the chief source of the B vitamins used in biological in-

TABLE VIII

GAINS IN BODY WEIGHT AND FOOD INTAKE OF GROUPS OF RATS GIVEN DIETS CONTAINING 18, 50, AND 90 PER CENT OF CASEIN SUPPLEMENTED BY DIFFERENT LEVELS OF YEAST

Group	Mean gain in weight	Significance ratio	Food calories ingested*	
			Total	Significance ratio
A1	gms. 128		2308	
A2	143	3.53	2483	2.5
A3	129	0.26	2414	1.6
P1	128		2091	
P2	135	1.42	2222	5.7
P3	133	0.88	2338	7.2
Z1	100		1910	
Z2	113	2.87	2033	1.6
Z3	97	0.76	2086	2.1
KA1	128		2724	
KA3	119	1.58	2509	2.9
KZ1	107		2056	
KZ3	110	0.12	2197	3.1
Z2	113		1903	
Z3	97	2.93	1959	0.7

\* Total calories include those derived from yeast.

vestigations, a comparison is given in Table VIII of the growth-stimulating effects of the three levels used as supplements to each of the three rations. The groups which made the best gains in weight are A2, P2, and Z2. However, in the case of only the A2 group is there a significant difference in the weight gained over that attained by the other groups fed on the same level of protein (A1 and A3), but whose yeast allowance was different. It appears that the 0.8 gm. level of yeast is the best growth-stimulant of any of the quantities of yeast alone which we have used.

When, in the absence of yeast, either the control or the high protein ration was supplemented with a large supply of vitamin G from autoclaved yeast and a minimum of B from tikitiki extract (groups A6 and Z6, Table III), the growth-response of the rats was meagre. Likewise, if an excessive amount of B together with a very small allowance of G were furnished, the mean gains in weight were still poor. Results similar to these have been observed by Graham and Griffith (13). It is likely that in each case the vitamin which was given in smaller amount was inadequate in quantity, or that some other of the B vitamins necessary for growth either is not furnished at all, or is supplied in insufficient quantity under the conditions of this study.

There are many conflicting reports concerning the ability of rats to grow on high-protein rations in a normal manner or as well as their controls fed the ordinary levels of protein. In view of our results it seems possible that the lack of agreement may be due to a deficiency of one or more of the B vitamins.

It is quite possible that under paired feeding conditions our results would be quite different from those obtained when rats have free access to an abundant food supply. One of us (L. D. F.) is at present investigating this phase of the subject.<sup>4</sup> Data from a very limited number of rats at present point to a better utilization of the protein-rich food for the growth of rats, than of the ration containing 18 per cent of casein.

In a comparison of the caloric intake of our groups of experimental rats with that of their respective controls (Table V), the significance ratio figures in all but two cases indicate that the experimental animals consumed a significantly less amount of food. This comparison holds even with the A4 and Z4 groups, whose mean gains in weight are not significantly different. This condition suggests, as indicated above, that the two high-protein rations used, in terms of calories, are more efficient in the production of growth in young rats than is a diet containing 18 per cent of protein. A

<sup>4</sup> In the Physiology Laboratory of Wellesley College.



smaller energy intake on the part of rats confined to a protein-rich dietary, has also been observed by Smith and Moise (10).

### SUMMARY

Unilateral nephrectomy *per se* did not impair the ability of rats to grow when fed a ration containing any one of the three given levels of casein.

Rats restricted to a diet containing 50 per cent of casein grew as well as their controls having 18 per cent of this protein in their rations and better than rats confined to a 90 per cent casein-containing dietary.

Only 28 per cent of the groups maintained on the food containing 90 per cent of casein made gains in weight equal to those of their controls which had 18 per cent of casein in their ration.

Of the five groups of rats fed the highest level of protein supplemented with yeast alone, 80 per cent of the groups made inferior gains in weight in comparison with their controls and all were inferior to the groups of rats whose diet contained 50 per cent of casein.

The optimum level of dietary protein for the production of rapid growth, appears to lie between 18 and 50 per cent.

The best growth-promoting supplement, when yeast was used alone, was 0.8 gm. This quantity fed with the control diet produced significantly better growth than either the 0.4 or the 1.6 gm. level. With the rations containing more protein, however, any one of the levels of yeast served equally well for stimulating growth.

The best growth attained on the ration containing 18 per cent of casein was by the use of 0.8 gm. of yeast and also by the 1.6 gm. level of yeast plus tikitiki extract.

The best growth recorded for the highest protein diet occurred when the 1.6 gm. level of yeast was fortified with either tikitiki extract or with autoclaved yeast.

In the absence of yeast, both an excess of B with a minimum of G, or an excess of G along with a small amount of B are unsatisfactory growth stimulants. Probably autoclaved yeast and tikitiki extract furnish only some of the necessary B vitamins.

There is some evidence that better utilization of food for growth is possible by feeding high protein rations in contrast to the control diet containing 18 per cent of casein.

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# CALCIUM AND PHOSPHORUS IN THE DEVELOPMENT OF THE TURKEY EMBRYO\*

By

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PROGRESS in the determination of causes of embryo mortality seems to be dependent in a measure upon accurate knowledge of the metabolism of the developing embryo. A review of the literature of this subject is given by Needham (1).

## EXPERIMENTAL METHOD

The Bronze turkey eggs used in this experiment were produced at the Western Kentucky Experiment Substation at Princeton, Kentucky. They were collected for a period of thirteen days, shipped to Lexington, Kentucky, and placed in an electric (Petersime) incubator. The average temperature was 99.75°F. throughout the period of incubation with a relative humidity of 64 per cent. All eggs were turned four times daily. At twenty-four hour intervals, six eggs weighing 80 to 90 grams each were removed and stored in a refrigeration room at 28 to 30°F. This effectively stopped embryonic development and preserved the eggs until analyzed at a later date. The embryos were removed from the eggs, compared with previously standardized specimens, freed of all adhering yolk, including that within the body cavity, dried on filter paper, transferred to silica dishes, weighed, and dried to a constant weight in an electric oven at 100C.° The dry embryos were burned to a white ash, which was dissolved in hydrochloric acid, made to a definite volume with distilled water, and calcium and phosphorus determined in separate aliquots. Calcium was determined by the McCrudden volumetric method (2) and phosphorus by the volumetric method of the Association of Official Agricultural Chemists (3).

## RESULTS AND DISCUSSION

The present study of the Bronze turkey embryo is concerned with: 1.—embryonic growth as measured by wet weight, dry weight, and ash content; 2.—percentage of moisture; 3.—growth cycles; and 4.—calcium

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\* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

and phosphorus content. Comparisons of these studies will be made with those reported on the chick embryo.

The data covering the growth of the turkey embryo as measured by wet weight, dry weight, per cent moisture, and ash content, are given in Table I and Figure 1. The daily increase in wet weight, dry weight, and ash con-

TABLE I  
GROWTH, MOISTURE AND ASH CONTENT OF THE TURKEY EMBRYO

Day	Average weight of eggs	Number of embryos	Wet weight grams	Dry weight grams	Per cent moisture	Ash content grams
5	84.2	5	.0156	.0050	67.95	
6	84.5	2	.0330	.0104	68.48	
7	83.5	4	.0797	.0204	74.40	.0004
8	83.7	3	.1903	.0279	85.34	.0013
9	84.5	2	.5121	.0586	88.56	.0044
10	86.3	3	.5823	.0576	90.11	.0043
11	82.5	2	.9492	.0719	92.43	.0085
12	82.5	4	1.7670	.1583	91.04	.0138
13	83.0	5	3.9664	.2719	93.15	.0269
14	82.7	6	4.4310	.3715	91.62	.0319
15	83.7	3	5.6975	.6530	88.54	.0523
16	85.6	5	6.2538	.9141	85.38	.0669
17	84.8	6	7.0468	1.2354	82.47	.0865
18	81.0	2	12.1482	2.2035	81.86	.1501
19	83.7	3	15.4144	3.1686	79.44	.1923
20	84.5	4	16.5000	3.2900	80.09	.2175
21	83.5	4	23.8900	4.8200	79.81	.3250
22	84.0	4	27.5100	5.8200	78.86	.3950
23	85.8	5	33.2100	6.8600	79.35	.4900
24	83.4	5	36.9300	7.8900	78.63	.5840
25	85.0	5	44.3600	9.2900	79.05	.6900
26	84.2	5	49.3600	11.3700	76.96	.7920

tent is very small until the twelfth day after which the increments of growth noticeably increase. As shown in Figures 1 and 2, embryonic growth is divided into three distinct phases or cycles with definite periods of retardation between the ninth and tenth days and between the nineteenth and twentieth days. These periods of retardation appear at approximately the same relative time as those appearing on the ninth and sixteenth days for the chick embryo. Needham (*loc. cit.*) has shown that during the early period of incubation up to and including the seventh day, the chick embryo uses carbohydrate as the source of energy. This period corresponds to the first nine days of the turkey embryo's life and it will

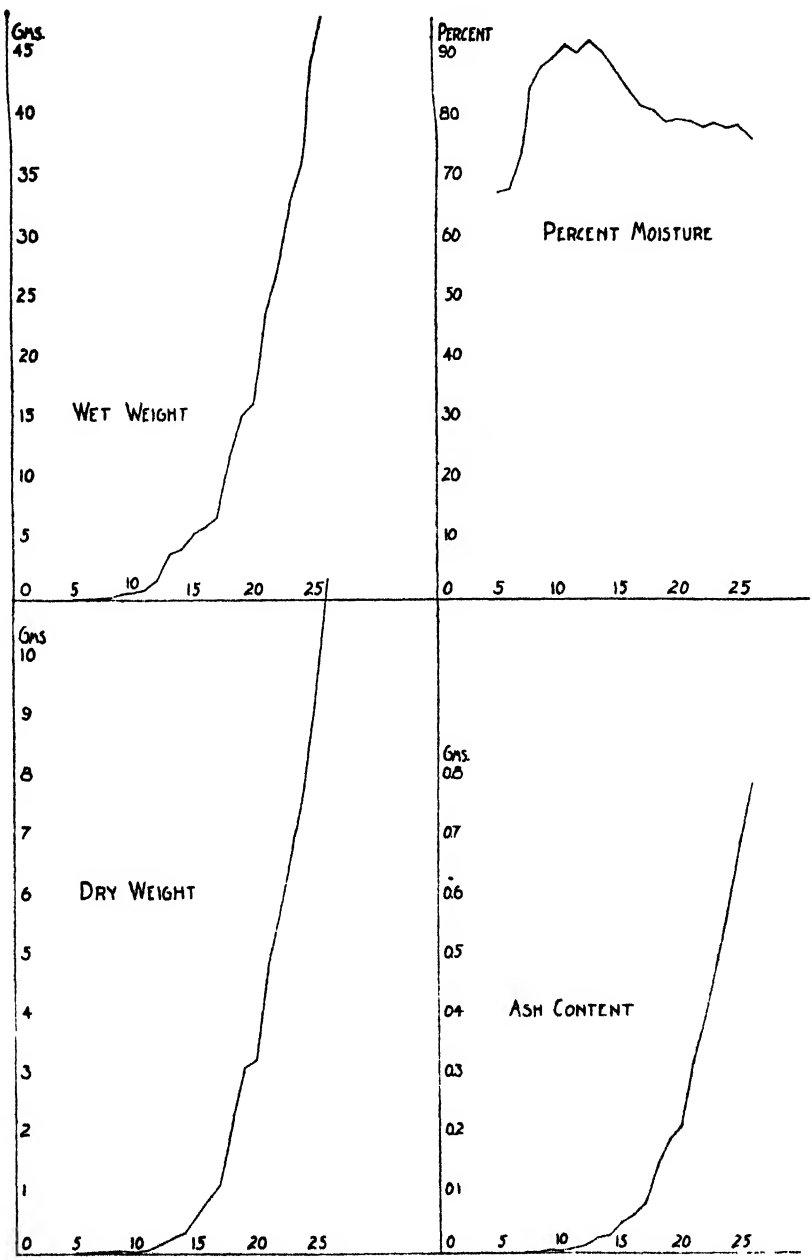


FIG. 1.—The Growth of the Bronze Turkey Embryo as Measured by: Wet Weight, Ash Content and Per Cent of Moisture.

be noted that the retardation of growth between the ninth and tenth days probably marks the limit of this phase of embryonic metabolism. It is especially noticeable that the percentage rate of gain in dry matter (Figure 2) is 71.0 from the eighth to the ninth day and -1.7 per cent from the

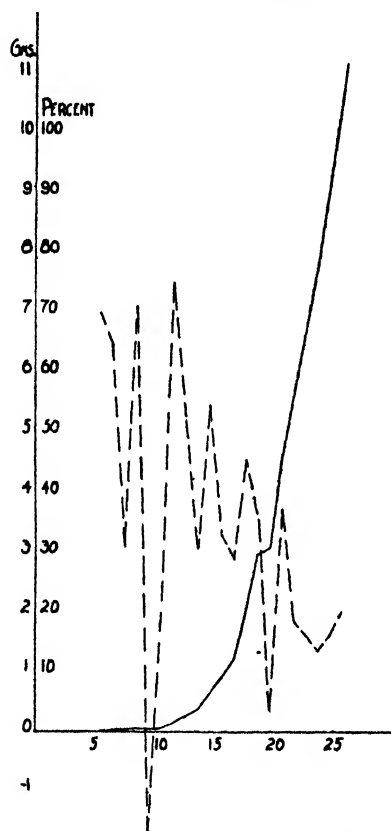


FIG. 2.—Cumulative Gain and Percentage Rate of Gain Turkey Embryo.

ninth to the tenth day. The formula used for the calculation of the percentage rate of growth was a modification of Minot's formula proposed by Brody (4). Percentage rate of gain =  $100 \frac{(W_2 - W_1)}{\frac{1}{2} (W_1 + W_2)}$  in which  $W_1$  = weight at the beginning of the period and  $W_2$  = the weight at the end of the period.

The second period of retardation which occurs between the nineteenth and twentieth days gives a definite decrease in percentage rate of gain, and a decided pause in dry weight, wet weight, and ash content. Needham has discussed the utilization of protein during the period from the seventh

to the sixteenth day of incubation and the metabolization of fat thereafter. The break in the curve of the turkey embryo corresponds to the change in materials metabolized. This break in the curve of the chick coincides with the peak of embryonic mortality as shown by Payne (5). Mortality curves have not as yet been given for turkey embryos.

The percentage of moisture in the turkey embryo was found to be the lowest between the fifth and seventh days of incubation. The moisture content at this period was approximately that of the content of the egg. The moisture content of the embryo increases rapidly from the sixth to the eleventh day of incubation and remains fairly constant from the eleventh to the fourteenth day. After this time the percentage of moisture declines steadily to the nineteenth day and remains rather constant until the twenty-fifth day.

The data on the calcium and phosphorus content of the turkey embryo are presented in Table II and Figure 3. The total amount of calcium gives a somewhat similar picture to that presented by the growth curves of the embryo. The increase in calcium content is relatively slow until the seventeenth day, when the growth becomes more rapid with the exception of the

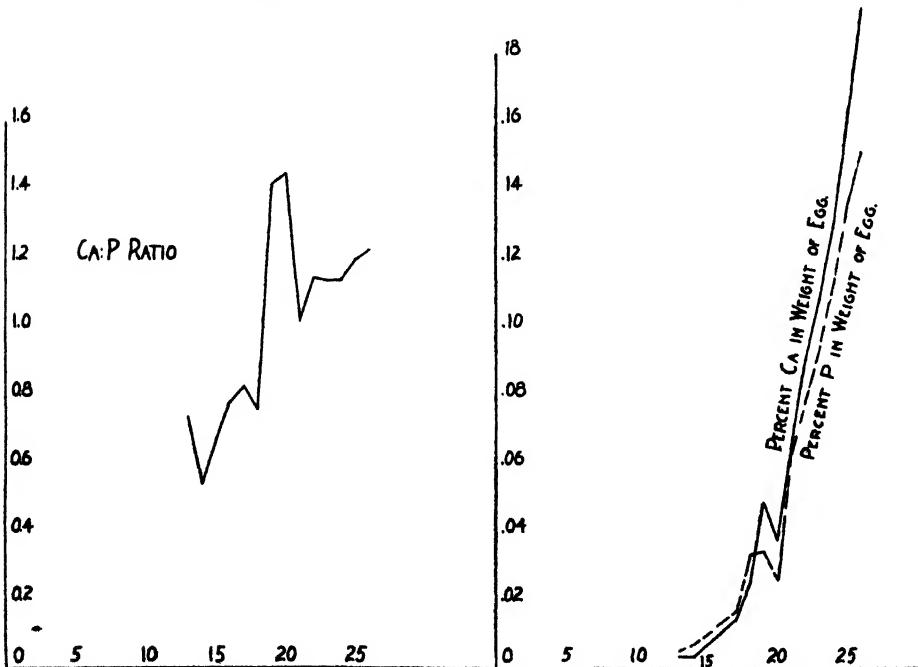


FIG. 3.—Calcium and Phosphorus in the Developing Turkey Embryo.



decrease from the nineteenth to the twentieth day. The rapid increase in calcium content is resumed after the twentieth day. The phosphorus curve follows very closely that of calcium, with the same decrease from the nineteenth to twentieth day. The calcium-phosphorus ratio with phosphorus remaining constant at 1.0 is shown in Figure 3 and Table II. Until the

TABLE II  
CALCIUM AND PHOSPHORUS CONTENT OF TURKEY EMBRYOS

Day	Calcium (Ca) grams	Phosphorus (P) grams	Ca:P ratio
13	.0031	.0042	.74:1
14	.0031	.0057	.54:1
15	.0057	.0085	.67:1
16	.0092	.0118	.78:1
17	.0119	.0143	.83:1
18	.0207	.0274	.76:1
19	.0409	.0289	1.42:1
20	.0321	.0222	1.45:1
21	.0543	.0534	1.02:1
22	.0764	.0664	1.15:1
23	.0928	.0812	1.14:1
24	.1081	.0948	1.14:1
25	.1388	.1160	1.20:1
26	.1632	.1327	1.23:1

nineteenth day the value of calcium is below 1.0 while after that time the value is more than 1.0. The calcium-phosphorus ratio during the latter period of incubation (approximately 1.2 Ca:1.0 P) may be an indication of the proper ratio in the diet of newly hatched turkeys.

The ratio between the calcium and phosphorus in the embryo may suggest the manner in which these elements are combined as the embryo develops. For example, the ratio between the calcium and phosphorus in monocalcium phosphate ( $\text{CaH}_4\text{P}_2\text{O}_8$ ) is 0.65:1. This closely approximates the ratio in the embryo up to the nineteenth day. On and after the nineteenth day the ratio is more nearly that of dicalcium phosphate ( $\text{CaHPO}_4$ ) or 1.29:1. In this connection it is interesting to note that Romanoff and Romanoff (6) found a marked lowering of the pH value of egg yolk on the sixteenth day of incubation of the hen's egg. This corresponds approximately to the nineteenth day of the life of the turkey embryo. This abrupt change from an alkaline to an acid state and the return to alkalinity is explained by them as follows, "The above-mentioned sudden drop in pH

value under both natural and artificial incubation is possibly related to the natural depression of growth in the life span of the embryo." It is possible, however, that this change of pH is ascribable to a sudden change in the form of the calcium-phosphorus compound present, as discussed above.

#### SUMMARY

1. A study was made of 1.—embryonic growth as measured by wet weight, dry weight and ash content; 2.—per cent of moisture; 3.—growth cycles; 4.—calcium and phosphorus content of the Bronze turkey embryo.

2. The daily increase in wet weight, dry weight, and ash content was very small until the twelfth day, after which time increments of growth noticeably increase.

3. The increase in calcium and phosphorus content was relatively slow until the seventeenth day.

4. The calcium-phosphorus ratio was less than 1.0 until the nineteenth day of incubation, after which time it exceeded 1.0.

5. Turkey embryo growth is divided into three distinct phases or cycles, with definite periods of retardation between the ninth and tenth and between the nineteenth and twentieth days. These periods of retardation were also noted in the calcium and phosphorus content of the embryo. Certain correlations between these periods of retardation and the change in the type of food metabolized are suggested.

6. It is suggested that the calcium content of the embryo may be used as a measure of growth provided the yolk material drawn into the body cavity is removed before analysis.

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# THE INFLUENCE OF THE PRECEDING DIET UPON THE RATE OF GLUCOSE ABSORPTION AND GLYCOGEN SYNTHESIS

By

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CARBOHYDRATE tolerance, as measured by the height of the blood sugar curve following the ingestion of a given quantity of glucose, is varied by the nature of the preceding diet (1-8). The tolerance is lower after fasting than when food has been taken, reduced by a high protein or a high fat diet, and increased by a diet high in carbohydrate. The influence of the preceding diet upon the glucose tolerance may be either through an effect upon the rate of glucose absorption from the intestine, the rate of hepatic and muscle glycogen synthesis, the rate of glucose absorption by the tissues (with storage, oxidation or conversion to fat), or all of these factors. The present experiments were designed to determine the influence of the preceding diet upon the first two of these factors in the albino rat.

## METHODS

Male albino rats of very nearly the same age served as experimental subjects. Three groups of 42 rats each were fed for 45 days on the experimental diets described in Table I. Half of these in each case were starved for 24 hours and the other half were starved for 48 hours. Six rats of each group of 21 served as controls. Fifteen were each given 1.0 cc. 50 per cent glucose solution. Three of the controls and three receiving glucose were killed at the end of one hour, being given amytal. Groups of three each were killed at the end of a 2, 3, 4, and 5 hour period and the remaining three controls were also sacrificed at this time. The rate of glucose absorption from the intestine was thus determined by Cori's method (9). The rate of glycogen deposition in the liver was also measured by the technic described by Cori (10). When the rats were anesthetized a sample of blood was collected for a sugar determination. The sugar content of the blood and intestinal contents was determined by the Shaffer-Somogyi modification (unpublished). The liver specimens were frozen in an ether-carbon dioxide snow mixture and weighed while frozen. Skeletal muscle specimens

TABLE I

Ingredients	Diet 1	Diet 2	Diet 3
Commercial casein	65	15	15
Raw cornstarch	0	50	0
Powdered yeast (Fleischmann's)	10	10	10
Salt mixture (Osborne and Mendel)	4	4	4
Lard	15	15	37
Cod liver oil	6	6	6
Cellulose (powdered)	0	0	28

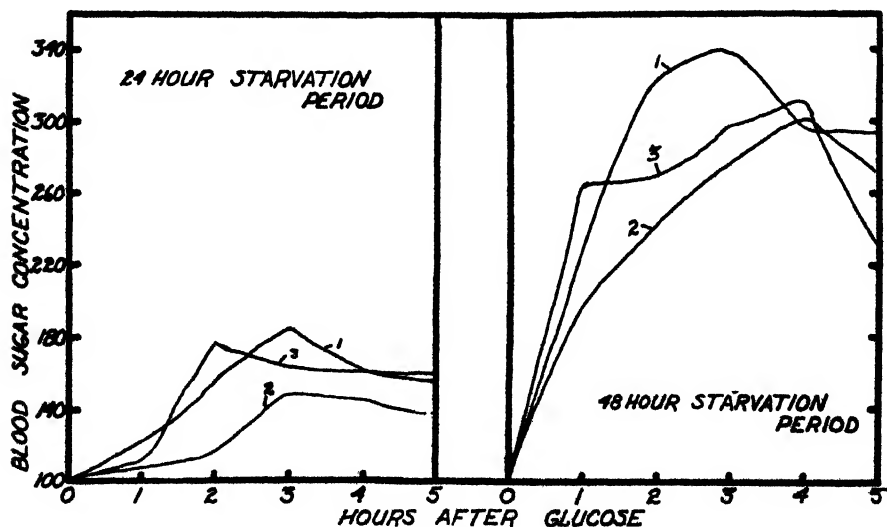
*Composition*

Protein	65	17	17
Carbohydrate	22	22	44
Fat	4	50	4

*Proportion of Calories*

Protein	56	14	14
Carbohydrate	2	43	3
Fat	42	43	83

were rapidly removed from the lower limbs and treated in the same manner. The glycogen samples were received into boiling KOH solution and glycogen determined by Sahyun's method (11).



FIGS. 1 AND 2.

TABLE II

Length of absorp. period hr.	24 hours starvation						48 hours starvation							
	Body surface sq. cm.	Liver weight gm.	Liver glycogen per cent	Muscle glycogen per cent	Glucose fed gm.	Glucose in G. I. tract gm.	Blood sugar conc. mg. per cent	Body surface sq. cm.	Liver weight gm.	Liver glycogen per cent	Muscle glycogen per cent	Glucose fed gm.	Glucose in G. I. tract gm.	Blood sugar conc. mg. per cent
<i>Diet 1—Protein</i>														
0	485	8.4	0.20	0.18	0.00	0.00	93	391	6.3	0.45	0.22	0.00	0.00	68
1	549	9.5	0.12	0.19	2.75	1.65	112	396	5.8	0.88	0.21	1.98	1.31	171
2	544	9.1	1.41	0.21	2.62	1.38	154	417	7.1	1.46	0.27	2.08	1.53	236
3	527	9.2	2.40	0.25	2.64	1.35	185	398	6.1	2.17	0.33	1.99	0.80	247
4	533	9.7	2.28	0.30	2.96	1.39	164	401	6.8	2.84	0.26	2.01	0.63	222
5	572	10.0	2.27	0.32	2.76	0.71	163	388	7.1	3.33	0.42	2.01	0.43	215
0	577	8.5	0.15	0.19	0.00	0.00	105	373	5.4	0.46	0.18	0.00	0.00	78
<i>Diet 2—Carbohydrate</i>														
0	543	8.9	1.05	0.21	0.00	0.00	124	417	6.6	0.18	0.21	0.00	0.00	53
1	534	10.4	0.97	0.18	2.67	1.55	141	403	6.3	0.62	0.25	2.02	1.05	101
2	515	10.3	1.99	0.26	2.58	1.43	141	423	6.5	0.88	0.24	2.03	1.16	119
3	434	8.3	2.09	0.31	2.34	1.22	180	427	7.2	2.58	0.27	2.01	1.13	138
4	508	9.6	2.89	0.33	2.67	0.55	157	428	6.3	2.64	0.22	2.14	0.81	158
5	499	8.8	3.14	0.38	2.67	0.55	157	428	6.6	3.28	0.30	2.15	0.47	135
0	486	8.4	0.71	0.21	0.00	0.00	113	369	5.2	0.09	0.23	0.00	0.00	47
<i>Diet 3—Fat</i>														
0	547	10.0	0.88	0.29	0.00	0.00	106	425	6.0	0.29	0.17	0.00	0.00	73
1	510	10.3	1.08	0.28	2.56	1.62	117	423	6.1	0.55	0.17	2.11	1.61	177
2	541	12.7	1.25	0.34	2.70	1.47	188	422	6.5	1.58	0.31	2.11	1.19	179
3	514	10.2	2.13	0.40	2.57	1.19	172	415	6.9	2.21	0.40	2.07	0.80	195
4	548	11.3	2.66	0.42	2.73	1.51	170	396	6.4	2.48	0.17	1.95	0.81	199
5	498	9.9	3.05	0.52	2.49	0.79	166	449	7.8	3.23	0.44	2.25	0.52	152
0	531	9.1	0.39	0.24	0.00	0.00	107	398	6.2	0.13	0.23	0.00	0.00	59



## RESULTS

The summarized figures presented in Tables II and III are all averages for three rats. The blood sugar curves (Figs. 1 and 2) after glucose following the various diets show relations which might be expected (4).

Cori (9) used as his absorption coefficient the amount of sugar absorbed per hour in relation to body weight. We have found (12) that the absorption rate bears a more constant relation to body surface than body weight. Consequently the rate of glucose absorption per 100 sq. cm. body surface per hour has been used as the absorption coefficient. Cori (9) found the absorption coefficient constant from hour to hour, a straight line relationship. We find a marked falling off in the absorption rate during successive hours after giving glucose, a finding which will be discussed in more detail elsewhere.

The glucose absorption coefficient (Table IV) is highest after the carbohydrate diet and lowest after the high fat diet. It is obvious that changes in the absorption rate can hardly contribute to any variation in the glucose tolerance curve, but on the contrary make it necessary for some other factor to over-compensate the changes in the absorption rate. Our figures confirm Cori's (13) observation that the absorption coefficient for glucose tends to be lower after a 48-hour starvation period than after 24 hours.

TABLE IV

AVERAGE ABSORPTION COEFFICIENTS (AMOUNT OF SUGAR (GM.) ABSORBED PER 100 SQUARE CENTIMETERS BODY SURFACE PER HOUR)

Hours after food	All absorp. periods		First single hour absorption period		All absorp. periods except first single hour	
	24	48	24	48	24	48
Diet 1 Protein	0.100	0.100	0.201	0.169	0.080	0.084
Diet 2 Carbohydrate	0.116	0.114	0.210	(0.241)	0.093	0.082
Diet 3 Fat	0.102	0.096	0.184	0.118	0.082	0.090

The average fasting glycogen content of skeletal muscle (Table V) after the various diets shows no notable change with the possible exception of the high figure for the six fat fed rats after 24 to 29 hours of starvation. The figures for this group were uniformly high.

The fasting liver glycogen in Table V was naturally highest in the high carbohydrate group after 24 hours starvation. The fat-fed rats had more glycogen in their liver than the high-protein-fed ones, just the reverse of



TABLE V  
FASTING GLYCOGEN CONTENT

Hours without food	Skeletal muscle per cent			Liver per cent		
	24-29	48-53	Av.	24-29	48-53	Av.
Diet 1 Protein	0.18	0.20	0.19	0.17	0.45	0.31
Diet 2 Carbohydrate	0.21	0.22	0.21	0.88	0.13	0.51
Diet 3 Fat	0.26	0.20	0.23	0.63	0.21	0.42

findings reported (14) on rats receiving very similar diets without any starvation. On further starvation both the fat and carbohydrate fed rats lost glycogen while the protein fed rat liver apparently gained it. This may have been partly the result of the loss of water (14).

Hepatic glycogen was synthesized (Table III) just about as rapidly on all three diets after 24 hours starvation. The rate was lower if anything in the high carbohydrate group, just the reverse of what we had expected. This may be due to the higher fasting (24 hours) liver glycogen content of these livers for after the administration of glucose after a 48 hour fasting period (Table III) glycogen synthesis was most rapid on the high carbohydrate diet group. The concentration of glycogen, though, was practically the same in all three groups at the end of the five hour absorption period.

The changes in the glycogen content of skeletal muscles were not of such a degree that they might have any significant influence upon the blood sugar tolerance curves.

#### CONCLUSIONS

We have found no influence of the preceding diet upon either the rate of glucose absorption from the intestine or the rate of glycogen synthesis in either the liver or skeletal muscle of such a nature that it might account in any degree for the variations in the blood glucose tolerance curves produced by diet. Consequently we must conclude that these variations are produced almost if not entirely by changes in the rate of removal of glucose by the tissues. Our experiments do not show definitely the mechanism which leads to this variable rate of glucose uptake by the tissues which may be due to changes in storage of the sugar as such, as glycogen, or in the rate of oxidation. We know that storage as glycogen in the tissues (skeletal muscle) is unimportant and it seems unlikely that there could be such continued differences in the rate of glucose storage as such. Changes in the rate of glucose oxidation seem by far the most likely explanation.

The current view that the influence of the preceding diet (8) upon the blood sugar tolerance curve is due to the level at which the sugar metabolizing hormone has been produced and hence is available (e.g., a high carbohydrate diet would call for more insulin than fasting and after it a lower glucose tolerance curve would result) fits in well with this picture. Insulin is known to be without influence upon the rate of hepatic glycogen deposition in the normal animal (15) and there is no evidence that it may affect the rate of absorption of glucose from the intestine.

### SUMMARY

In the albino rat the preceding diet, as varied by being composed chiefly of protein, carbohydrate, or fat, was without significant effect, after 24 and 48 hours of starvation, upon the rate of absorption of administered glucose from the intestine or the rate of glycogen deposition in the liver or skeletal muscle. It is therefore concluded that the variations in the blood glucose tolerance curve produced by the preceding diet are due in large part, if not entirely, to changes in the rate of glucose uptake by tissues other than the liver probably produced by variations in the rate of glucose oxidation.

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## THE POSSIBILITY OF GLUCONEOGENESIS FROM FAT

### II. THE EFFECT OF HIGH FAT DIETS ON THE RESPIRATORY METABOLISM AND KETOSIS OF MAN\*

By

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#### INTRODUCTION

SHOULD fatty acids in considerable quantity be transformed to carbohydrate and the latter remain unoxidized for some time in the mammalian body, the effect inevitably would be a depression of the respiratory quotient below the level of ordinary fasting. Quotients above unity indicating the reverse reaction are easily obtained, but the rarity of very low quotients, say below 0.67, reliably reported, indicates that it certainly is difficult to induce this transformation. Obviously it was to the advantage of primitive man as well as to the beasts in a state of nature that fat should be formed more easily than resolved again. Nature is a better banker than man. Spending from savings and the conversion of credits (fat) to small coin (sugar) should be difficult, if not impossible.

Ordinarily the laying on of fat takes place very gradually and the R.Q. need not exceed unity. Perhaps the reverse process also takes place very little at a time, short periods of conversion alternating with short periods of combustion, or, possibly, the two running concurrently, so that the details are obscured. It is evident that if combustion kept up with conversion there would be no lowering of the R.Q. This situation has long been recognized, but so far as we can discover, nobody has made any attempt avowedly to devise conditions wherein the two component reactions might conceivably be separated in time sufficiently for independent recognition. One of us has shown (1) recently that this is possible both temporally and spatially with a fatty seed (castor bean) where sugar is formed from fat in the endosperm and oxidized (in part) in the young plant.

In a student problem in nutrition one of the authors (J) appeared to have an unusual tolerance for fat. This observation coincidentally was

\* A large part of the data in this paper was contained in a thesis submitted by Estelle E. Hawley to the University of Rochester in partial fulfillment of the requirements for the Ph.D. degree.

made, as Dr. I. McQuarrie was carrying out in this laboratory analyses of respired air from epileptic children on ketogenic diets, in which some quite low respiratory quotients were observed. It occurred to us that by prolonging a tolerable, high fat diet for several days it might be possible to induce a condition of glycogen hunger, with no other adequate means of supply except a sudden, great excess of soluble fatty acids, and that in this combination of circumstances the tendency would be first toward conversion of fat to sugar (low respiratory quotient<sub>s</sub>) and sometime later toward combustion (high quotients). Maintenance of partial undernutrition would serve to deplete the glycogen stores. The tissues, liver in particular, would lay first claim to any sugar formed and would gradually pay out its gain to the muscles and other organs after the first full flood of fat absorption had passed. This was the hypothesis. Easily digestible and assimilable fat which contained a wide distribution of fatty acids would be the one of choice. Just what level of fat ingestion and what fatty acid-to-glucose ratio in the food would be most favorable to the purpose remained to be seen. The occurrence of such a sequence of quotients would not of itself prove the conversion of fat to carbohydrate, unless there were no other plausible explanation.

#### PROCEDURE

The first experiment occurred in January and February, 1930, with two subjects, H and J, who were at the same time the operators, each for the other, of the respiration apparatus (Benedict Universal). They took for 6 days a diet containing 74 per cent of the calories as fat (F.A.:G by weight = 1.5). This was followed immediately by a diet of the same general character containing 84 per cent fat (F.A.:G by weight = 3.0) for 7 days and this, in turn, by a third with 94 per cent fat (F.A.:G = 4.1) for 5 days. The last diet consisted exclusively of 4X cream (actually 37.5 per cent fat by analysis).

A second experiment was carried out by these two subjects in April and May, 1930. The intermediate diet was maintained as before. A third subject (M) participated on the 4.1:1 ratio diet only.

The third experiment occurred in October and November when the high-ratio diet was taken again by subjects M and J; and in February, 1931, it was taken by a fourth subject (V.S.) who, while on the diet submitted twice to a shivering experiment, which proved especially interesting from the standpoint of the respiratory quotient. In November, 1931, a student (W) volunteered to take the high-fat diet and was studied by one of us using the Tissot-Haldane procedure (Chart 4). Finally in November, 1932,

another student (F) took the all-cream diet and was studied, under supervision, by another student competent in air analysis procedure (Chart 4).

Aside from the respiratory quotient it was, of course, necessary for purposes of interpretation to have knowledge of the acidosis, if any, produced and any indication of conversion of fat to carbohydrate which might be obtainable from analysis of the blood. A rise of blood sugar, should it occur, ought not to be missed. Accordingly in three subjects total and ammonia nitrogens and acetone bodies in two fractions were determined in the urine and acetone bodies,  $\text{CO}_2$ -combining power (occasionally), total fat, and blood sugar, all in whole blood. Both urine and blood were collected at three-hour intervals, the collection in both cases coming immediately, or soon after the termination of a respiratory determination. Most complete data were obtained for the second and third experiments on the 4.1:1 ratio diet and consequently the discussion will concern this diet chiefly.

The daily routine for the subject who had been on diet for several days, typically was as follows on the experiment days:

1. Bladder emptied on arriving at laboratory.
2. One-half to one hour rest on bed.
3. Basal R.Q.'s immediately after which basal bloods were taken.
4. Basal urine collected.
5. High-fat test meal eaten (usually before 10.00 a.m.).
6. R.Q.'s at  $1\frac{1}{2}$  hour intervals from time of finishing meal.
7. Blood and urines collected at 3-hour intervals.

The majority of the experiments ran to 6 or  $7\frac{1}{2}$  hours, a few to 9 and two to  $10\frac{1}{2}$  and 11 hours.

*Methods of analysis.*—Total N and ammonia N in the urine were determined by the macro Kjeldahl and Folin aeration, respectively, urine and blood acetones by the Behre-Benedict (2) distillations and Hubbard's (3) titration methods. Blood sugar by Folin-Wu, blood fat by Bloor's (4) oxidation method and  $\text{CO}_2$ -combining power by the Van Slyke and Neill's (5) manometric method.<sup>1</sup> The respiratory quotients were determined in the majority of the daily runs by the Benedict "Universal" method only. The apparatus was provided with a graphic spirometer for better control of the exact time for throwing of the valve and the oxygen was measured into the apparatus after saturation with moisture by a carefully calibrated wet-test meter. In several experiments the R.Q.'s were checked by parallel

<sup>1</sup> We are indebted to Dr. E. S. Nasset for these determinations.

determinations by the Tissot-Haldane procedure,<sup>3</sup> employing the du-Vigneaud (6) modification of the Haldane. The agreement was not always perfect, but in the majority of cases was within the range of variation for successive determination by the same method (see below). The former apparatus was checked frequently by burning alcohol in the circuit and the latter by outside air analyses. No respiration experiment with the Benedict apparatus has been included for which there was not a graphic record of the respirations, showing that the valve was thrown correctly, *and a satisfactory alcohol check*. No experiment by the Tissot method is included, for which there was not a satisfactory outside-air analysis, checking the accuracy of the technic. These check data are omitted merely for conservation of space. A further check of importance to be noted is that most of the experiments on subjects H and J were carried out alternately by the same apparatus. A leaky apparatus could not give a low R.Q. on one subject and a high one on the other.

Altogether there were 32 experiment "days," meaning that respiratory quotients were taken for at least six hours following a test meal. On 18 "days" urine and blood analyses were made with more or less completeness.

## RESULTS

Space does not permit the presentation of more than a third of all of the tabular results of this study. For two subjects the following tables (I to IV) show the effects of a high and a low F.A. to G ratio at two different dates for each. The second low-ratio diet was taken about 11 weeks after the first and the second high ratio diet about 15 weeks after the first. For another subject three successive days are shown in Table V. These will illustrate the important factors concerned. Supporting data in addition will be found in the charts.

The low-ration diet was the same for subjects H and J. It was prepared in the diet kitchen of the department and consisted of three meals, except on experimental days, when only two were taken. Breakfast contained eggs, bread, butter, sugar, 4X cream and coffee; lunch, the same with the addition of peanut butter and orange juice; dinner, eggs, meat, milk chocolate, sugar, 4X cream, and coffee. The composition from standard tables was: protein 115, fat 286, carbohydrate 109 gms. The total fatty acid was calculated at 310.5 gms. and total glucose at 206.4, making the F.A.:G ratio 1.5. The total calories were too high.

<sup>3</sup> We are indebted to Dr. R. W. Swift for most of the

TABLE I  
SUBJECT H—RESPIRATORY METABOLISM AND KETOSIS  
F.A.:G ratio=1.5:1; 6th day of diet; meal contained 45 gm. F.A.

Date 1930	Time of period from meal	R.Q.	Liters per hr.		Heat per hr.		Per minute		Urine, mgrms./hr.			Blood values, mgrm./100 cc.		CO <sub>2</sub> comb. power vol. %
			O <sub>2</sub>	CO <sub>2</sub>	Total	Cal./sq. m.	Pulse	Resp.	Total N	NH <sub>4</sub> N	Total† acetone	Sugar	Total acetone†	
Jan. 19	Basal	0.76	11.24	8.54	53.4	35.1	76	14	356	15	10.8	92	42	—
	1½ hrs.	0.72	12.95	8.29	61.5	40.0	80	16	—	—	—	—	—	—
	3 hrs.	0.73	11.76	8.64	55.5	36.5	76	16	—	—	—	99	—	—
	4½ hrs.	0.74	11.84	8.80	56.0	36.8	80	16	—	—	—	—	—	—
	6 hrs.	0.73	11.34	8.23	53.4	35.1	76	14	—	—	—	91	39.5	—
	7½ hrs.	0.73	11.22	8.19	51.7	34.8	76	14	—	—	—	—	—	—
Apr. 8	Basal	0.69	11.58	7.97	54.3	35.7	66	9	329	41	17.9	82	14.4	53.2
	1½ hrs.	0.70	13.33	9.38	62.5	41.1	68	8	—	—	—	—	—	—
	3 hrs.	0.51*	13.65	6.96*	64.0	42.1	69	9	288	42	17.9	94	8.6	46.2
	4½ hrs.	0.70	13.04	9.16	61.1	41.1	74	8	—	—	—	—	—	—
	6 hrs.	0.68	12.65	8.60	59.3	39.0	69	8	436	38	19.8	95	13.6	51.6
	7½ hrs.	0.67	12.14	8.17	56.9	37.4	66	10	—	—	—	—	—	—

\* Probable error in weighing CO<sub>2</sub>—Heat computed for R.Q. of 0.707.

† "Total acetone" denotes the sum of preformed acetone, diacetic acid and  $\beta$ -hydroxybutyric acid expressed as acetone.



TABLE II  
SUBJECT J—RESPIRATORY METABOLISM AND KETOSIS  
F.A.:G ratio=1.5:1; 6th day of diet; meal contained 90 gm. F.A.

Date 1930	Time of period from meal	R.Q.	Liters per hr.		Heat per hr.		Per minute		Urine, mgms./hr.			Blood values, mgm. /100 cc.		CO <sub>2</sub> comb. power vol. %
			O <sub>2</sub>	CO <sub>2</sub>	Total	Cal./sq. m.	Pulse	Resp.	Total N	NH <sub>4</sub> N	Total acetone*	Sugar	Total acetone†	
Jan. 19	Basal	0.74	15.63	11.50	73.8	36.9	58	16	383	35	—	88	5.4	—
	1 hr.	0.66	18.58	12.19	87.1	45.8	60	16	—	—	—	—	—	—
	2½ hrs.	0.66	19.80	13.13	92.8	48.9	60	18	—	—	—	98	—	—
	4 hrs.	—	**	10.42	—	—	58	18	—	—	—	—	—	—
	5½ hrs.	0.67	16.59	11.12	77.7	40.9	60	14	—	—	—	91	5.9	—
Apr. 8	7 hrs.	0.73	15.63	11.42	73.7	38.8	63	16	—	—	—	—	—	—
	Basal	—	—	—	—	—	—	—	598	31	—	91	8.9	54.4
	1 hr.	0.75	20.85	15.61	98.8	52.0	62	18	—	—	—	—	—	—
	2½ hrs.	0.74	22.57	16.65	106.6	56.1	76	16	532	39	—	100	7.1	51.6
	4 hrs.	0.72	18.74	13.56	88.2	46.4	60	18	—	—	—	—	—	—
	5½ hrs.	0.72	17.12	12.40	80.6	42.4	56	20	600	41	—	107	9.2	51.8
	7 hrs.	0.74	16.93	12.60	80.1	42.2	70	18	—	—	—	—	—	—

\* Negative to ferric chloride.

\*\* Oxygen reading lost.

† "Total acetone" denotes the sum of preformed acetone, diacetic acid and  $\beta$ -hydroxybutyric acid, expressed as acetone.

TABLE III  
SUBJECT H—RESPIRATORY METABOLISM AND KETOSIS  
High fat; F.A.:G ratio 4.1:1 5th day of diet; meal contained 111 gm. F.A.

Date 1930	Time of period from meal	R.Q.	Liters per hr.		Cal. per hr.		Per minute		In urine, mgm. per hr.			In blood, mgm. per 100 cc.		
			O <sub>2</sub>	CO <sub>2</sub>	Total	Sq. m.	Pue	Resp.	Total N	NH <sub>4</sub> N	Total acetone	Sugar	Total acetone	Fat
Feb. 2	Basal	—	—	—	—	—	—	—	98	33	369	65	48	—
	1½ hr.	0.60	13.22	8.12	61.9	40.7	84	8	—	—	—	—	—	—
	3 hrs.	—*	—	—	—	—	—	—	—	—	—	—	—	—
	4½ hrs.	0.62	12.62	7.88	59.2	38.9	80	12	—	—	—	72	43	—
	6 hrs.	0.72	12.77	9.01	59.9	39.4	88	10	—	—	—	—	—	—
May 25	7½ hrs.	0.61	13.33	8.18	62.4	41.1	88	12	163	49	350	—	40.8	—
	Basal	0.64	11.81	7.61	55.3	36.4	72	8	197	71	196	67	35	498
	1½ hr.	—†	—	—	—	—	—	—	—	—	—	—	—	—
	3 hrs.	0.66	13.09	8.65	61.3	40.3	66	9	214	81	216	65	36	515
	4½ hrs.	0.67	13.32	8.92	62.4	41.1	70	9	—	—	—	—	—	—
	6 hrs.	0.68	13.69	9.27	64.2	42.2	72	11	216	80	206	74	28	†

\* Period omitted because of discomfort of subject.

† Period omitted to run alcohol check.

‡ Lost.

TABLE IV  
 SUBJECT J—RESPIRATORY METABOLISM AND KETOSIS  
 High fat; F.A.:G ratio 4.1:1; 5th day on diet; meal contained 222 gm. F.A.

Date 1930	Time of period from meal	R.Q.	Liters per hr.		Cal. per hr.		Per minute		In urine, mgm. per hr.			In blood, mgm. per 100 cc.		
			O <sub>2</sub>	CO <sub>2</sub>	Total	Sq. m.	Pulse	Resp.	Total N	NH <sub>4</sub> N	Total acetone	Sugar	Total acetone	Fat
Feb. 2	Basal	—	—	—	—	—	—	—	259	8	31.7	84	22.5	—
	1 hr.	0.75	21.41	13.10	101.5	53.5	76	16	—	—	—	—	—	—
	2½ hrs.	0.72	24.57	15.09	115.5	60.7	63	16	405	29	45.0	80	22.9	—
	4 hrs.	0.67	20.30	13.59	95.1	57.7	66	18	—	—	—	—	—	—
	5½ hrs.	—*	—	—	—	—	—	—	222	18	47.0	72	28.1	—
	7 hrs.	0.71	18.80	13.41	88.4	46.5	70	21	—	—	—	—	—	—
May 25	9 hrs.	—	—	—	—	—	—	—	175	27	63.0	—	26.9	—
	Basal	0.71	15.29	10.86	71.6	37.7	54	16	253	35	71.5	71	12.0	697
	1 hr.	—†	—	—	—	—	—	—	—	—	—	—	—	—
	2½ hrs.	0.74	20.46	15.06	99.6	50.9	62	19	307	38	80.7	71	39.0	837
	4 hrs.	0.72	19.37	14.03	92.2	48.0	66	20	—	—	—	—	—	—
	5½ hrs.	0.74	17.41	12.85	82.3	43.0	60	22	290	26	75.5	73	34.2	798

\* Subject fell asleep, ruining period.

† Period omitted to run alcohol check (see 1½ hr. on H., Table III).

In Table I may be seen contrasting effects, partly of the diet as a whole and partly of the single test meal, taken on the sixth day of the diet in each case, but taken in the second instance after the subject was well accustomed to the diet. The meal contained 45 gm. calculated fatty acid. The data for urine and blood are not so complete on the January experimental day as on the April day. To speak of the respiratory metabolism first, it is evident that the subject was more restless in the earlier experiment. The pulse and respiration indicate this, and possibly the R.Q.'s also. The principal cause was alimentary discomfort. There is evidence in the data for heat production that absorption was by no means so complete in the first experiment as in the second. The specific dynamic action was highest the first period (1½ hours) following ingestion of the meal in the former, but was maximal at the second period (3 hours) in the latter, and had not yet returned to basal at 7½ hours. The principal reason for the lower R.Q.'s in the second trial with this diet therefore would seem to be associated with the better utilization. The term applies to events beyond the alimentary wall as well as to those within; for while the figures for the urinary acetone bodies ("total acetone" throughout means all three acetone products combined and expressed as acetone) are higher in the later experiment, those for the blood total acetone are much lower both before and after the meal. Placing the greater emphasis on blood acetone, this subject illustrates the principle of adaptation in the sense of an acquired tolerance for a high-fat diet which has been noted by Wigglesworth (7) for the rat. It is seen also in Tables III and VI.

It is the more remarkable that the respiratory signs of altered metabolism (low R.Q.'s) should be so much more pronounced in the second trial. The CO<sub>2</sub>-combining power of the whole blood was lowest when the specific dynamic action was highest. There was no driving off of CO<sub>2</sub>. (It happened as a matter of ill-luck that the figure for CO<sub>2</sub> at the third hour was questionable, at the very time when, according to convention, it should have been high—the combining power at this point being lowest.) The ammonia figure for the urine indicates no change from basal at the 3-hr. collection; but it must be borne in mind that the urine represents the collective metabolism for the past three hours while the blood analyses indicate only a momentary condition.

There is a greater rise from basal in the blood sugar this (second) experimental day, but the meal contained a considerable amount of carbohydrate and therefore it probably is meaningless as evidence of gluconeogenesis. The same comment applies to the next experiment.

Subject J on the same general diet and following a test meal with twice the fatty acid value of that for subject H, on corresponding days of the diet, manifested a different reaction, at least as regards the R.Q.'s. (Table II.) They run just as uniformly high in the second trial as they did for subject H in the first; also they run even lower in the first trial than they did for subject H in the second. The specific dynamic action is in agreement with that of subject H in showing more rapid absorption the second, than the first trial, while the ketosis, so far as the record goes, is so slightly changed that very little can be said about it. Surely it does not help the interpretation of the R.Q.'s. Nor does the  $\text{CO}_2$ -combining power. The fall of a few volumes per cent following a fatty meal is something seen quite regularly in these experiments but it is seldom reflected in the respiration record in any way. Perhaps alveolar R.Q.'s after the manner of those recently reported by Carpenter and Lee (8) would be more significant, in a situation like this. There is nothing of interest in the other blood or urinary findings, except that the higher basal total urinary N the second trial may indicate some hang-over effect from the evening meal of the day before.

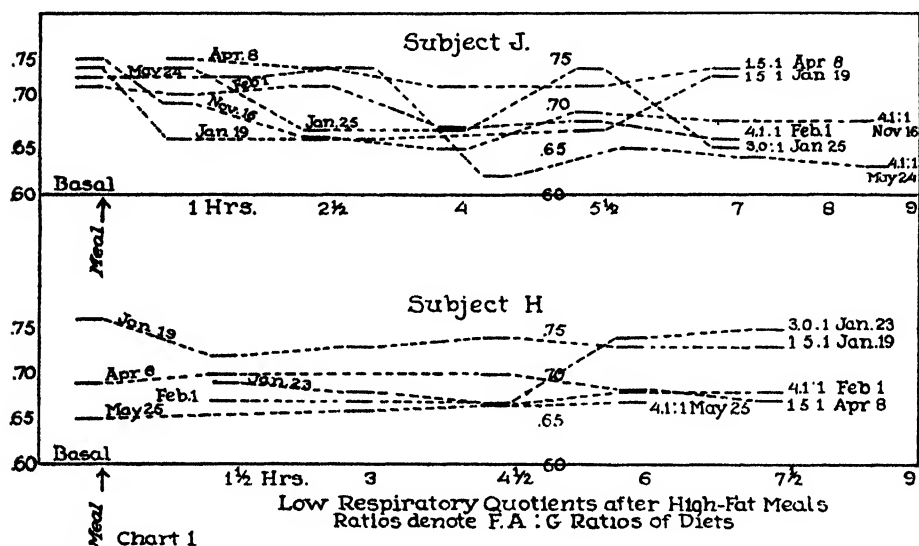
It should be emphasized again that the two experiments on H and J were run on the same day each time, alternating period by period on the same machine and since the operation of the apparatus also was identical for the two subjects, the differing results on the two cannot be accounted for by different conditions of the apparatus. Subject J also seemed to tolerate the diet and the test meal subjectively somewhat better the second time than the first. This is borne out by the continued low figures for ketosis. With subject H (a woman) the improved tolerance was reflected in the ketosis but not in the R.Q.'s with subject J decidedly more in the R.Q.'s. Since the test meal contained twice as much fatty acid equivalent for subject J, and there was at no time any significant production of ketone bodies, it is clear that the male subject had the higher tolerance for fat. This is in agreement with a recent report by Deuel and Gulick (9).

Immediately following the first low-ratio diet both subjects H and J took an intermediate diet having a F.A.:G ratio of 3:1 for 7 days and then the really high-ratio diet for five days. There were two experimental days, the 4th and 6th for each subject on the 3:1 diet, but for want of space they are omitted from the tables. Certain significant results from these days, however, are contained in Chart 1.

The same sort of comparisons for the first time and the second time on the high-ratio diet are seen in Tables III and IV. The test meal consisted exclusively of 4X cream. Again the days of the diet on which the metabo-

lism experiment was performed corresponded for the two subjects, and the test meal of subject J was twice that of subject H in fatty acid equivalent.

The R.Q.'s for corresponding periods of the two trials were not so different as they were on the low-ratio diet. The respiratory data are regrettably incomplete. The diet was exceedingly hard to take for some subjects and the somnolence and lassitude consequent upon the ketosis are subversive of the utmost willingness in the world to coöperate. Furthermore, the two



subjects were alternately active and quiet in serving as observer and subject (see page 526) which probably accounts for some irregularities. With subject H (Table III) the ketosis was again much greater than with subject J (Table IV), notwithstanding the much smaller meal of cream. The high basal figures for urinary and blood acetone indicate that the allowance of fat was much greater than the former subject could properly metabolize. Nevertheless she stuck to the regimen and in the second trial again achieved a measure of adaptation, as shown by the lower ketosis. There was less ammonia available for neutralization of the ketosis in the first trial than in the second, possibly because of somewhat better absorption of the proteins of the cream in the latter. The specific dynamic action, perhaps also as a consequence of the better absorption, was higher in the absolute sense the second trial. The exact basal heat production not being known the first trial, the percentage increase expressing the specific dynamic action cannot be given. In both instances absorption seems to have

been quite slow, for the heat production at the end of the day was greater than at the third or second periods. On the low-ratio meal (Tables I and II) the highest effect came at the first or second period, for both subjects.

Subject J (Table IV) seems to have handled the high-ratio diet and test meal better the first trial than the second. The basal urinary ketosis was low initially the first time, increased up to the fourth period only 50 per cent, and finally at nine hours to 100 per cent, while the blood ketosis scarcely increased at all. In the second trial the urinary ketosis started high basally, and scarcely increased following the meal up to 5½ hours, while in the blood the basal figure was very low and, following the meal, the value increased to 300 per cent and more.

The specific dynamic effect of the meal evidently was much greater in the first trial than in the second, and one is led to wonder whether the lower ketosis in the former had something to do with this result.

Judging by the R.Q.'s, subject J was able to burn butter fat nearly as rapidly as it could be absorbed; for, following the high-ratio meal they were, with only one exception, very close to the theoretical for this fat (0.72). An attempt will be made in the general discussion to account for the low quotients. At this point it will suffice to say that there seems to be no proportionality between any of the respiratory quotients and the level of ketosis whether this be taken from the cumulative figures of the urine or the periodic figures of the blood. The blood fat in the second trial with subject J runs a course roughly parallel with the heat production.

Subject M was on the high-ratio (all-cream) diet for two five-day periods, one in May and the other in October. During the first period three successive respiratory studies were made on the 3rd, 4th, and 5th days of the diet (Table V). On the 4th and 5th days the ketosis was traced by urinary analysis rather completely, and on the 4th and 5th days quite completely also by blood analysis. Only one respiration period was lost (May 21, 2nd period following the meal).

On the first experimental day the lowest R.Q.'s (0.68) were found at the 1st and 3rd periods (1½ and 4½ hours following the test meal). The same is true of the second day (0.69 and 0.70) although in this instance the intermediate period showed a higher R.Q. (0.75). The third day none lower than 0.72, occurring at the 3rd and 6th hour, was recorded. On each of the three days the R.Q. dipped below the basal level soon after the meal and then rose progressively to 0.77 or 0.78 at the end of the day (see Chart 2). Note that with the exception of a single determination (3d hr.) on the second day the general levels follow the order of the days, i.e., first, lowest,

TABLE V  
SUBJECT M—RESPIRATORY METABOLISM AND ACETONE-BODY PRODUCTION  
High-fat diet; F.A.: G=4.1:1; 3rd, 4th, and 5th days of diet; meal contained 227 gms. F.A.

Date 1930	Time of period from meal	R.Q.	Liters per hr.		Cal. per hr.		Per minute		In urine, mgms. per hr.			In blood, mgm. per 100 cc.			CO <sub>2</sub> comb. power vol. %
			O <sub>2</sub>	CO <sub>2</sub>	Total	Sq. m.	Pulse	Resp.	Total N	NH <sub>4</sub> N	Total acetone	Sugar	Total acetone	Fat	
May 21	Basal	0.74	15.59	11.59	73.9	33.8	66	13	—	—	FeCl <sub>3</sub> +	—	—	—	—
	1½ hrs.	0.68	20.17	13.75	94.5	43.2	69	12	—	—	+	—	—	—	—
	3 hrs.	omitted	—	—	—	—	—	—	—	—	—	—	—	—	—
	4½ hrs.	0.68	18.84	12.80	88.3	40.3	69	14	—	—	—	—	—	—	—
	6 hrs.	0.72	18.50	13.24	86.9	39.7	80	14	—	—	+	—	—	—	—
May 22	7½ hrs.	0.78	17.14	13.30	71.8	37.4	78	12	—	—	—	—	—	—	—
	Basal	0.71	17.03	12.08	75.0	35.1	60	12	285	48	15.2	97	27	—	—
	1½ hrs.	0.69	19.34	13.21	90.8	41.4	60	13	—	—	—	—	—	—	—
	3 hrs.	0.75	19.71	14.74	92.2	42.2	78	12	447	30	18.0	—	—	—	—
	4½ hrs.	0.70	19.02	13.29	89.1	40.7	72	14	—	—	—	—	—	—	—
May 23	6 hrs.	0.73	19.21	14.08	90.5	41.3	75	14	384	33	60.0	81	18	—	—
	7½ hrs.	0.77	17.77	13.49	84.7	38.7	66	14	—	—	—	—	—	—	—
	9 hrs.	0.77	17.03	13.03	81.0	37.0	75	10	360	53	41.6	—	—	—	—
	Basal	0.74	17.57	13.03	83.1	37.9	90	10	285	49	15.2	91	15	372	43
	1½ hrs.	0.73	20.45	14.97	96.4	44.0	78	10	—	—	—	85	61.3	735	—
	3 hrs.	0.72	20.83	14.92	97.9	44.7	90	10	372	36	36.0	94	24.0	815	39
	4½ hrs.	0.73	20.05	14.65	94.5	43.2	87	12	—	—	—	80	34.0	764	—
	6 hrs.	0.72	20.02	14.42	94.1	43.0	86	12	333	61	62.0	70	28.0	728	—
	7½ hrs.	0.74	18.04	14.00	85.2	38.9	87	10	—	—	—	70	24.0	737	—
	9 hrs.	0.76	18.83	14.25	89.4	40.8	86	14	—	—	—	72	48.0	790	—
	10½ hrs.	0.78	18.34	13.22	85.9	39.2	86	14	250	50	60	—	—	—	—



second intermediate and third, highest. This particular feature will be commented upon in the general discussion. The averages for the 3 days were .716, .730 and .740.

The specific dynamic effect of the meal was greatest on the second and third experimental days at the end of three hours following the meal. This coincides with the highest blood fat on the one day when it was followed, and it is probable that the same would have been shown in the period which was lost on the first day. This agrees fairly well with subject J, whose highest specific dynamic action occurred at the second respiration period ( $2\frac{1}{2}$  and  $2\frac{3}{4}$  hours) following his all-cream test meal. The heat curve fell fairly regularly from this high point to the end of the day. (Compare with the record of specific dynamic action of dogs fed emulsified fat by Murlin and Lusk, 10.) Even at  $10\frac{1}{2}$  hours (May 23), however, it was still above the basal level for the day.

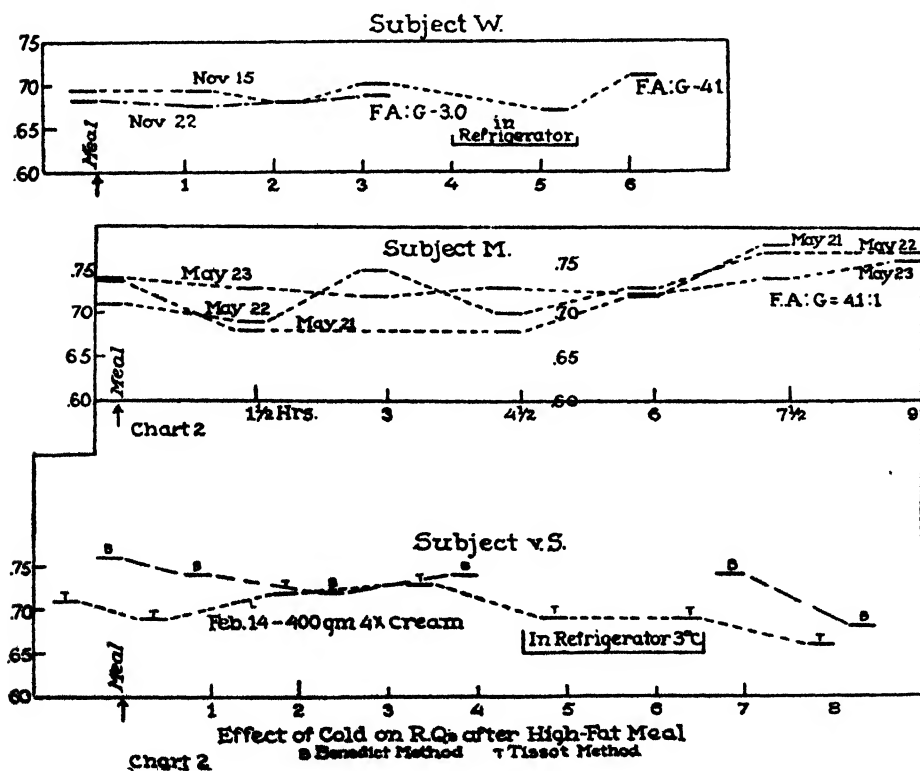
In the progressively higher basals on the 2nd and 3rd experimental days there is just a little evidence for the old idea of *luxus consumption*. But it might equally well be called simply a survival of the specific dynamic effect of the previous day's diet as a whole. It would be interesting to know how long it might have remained high.

The highest urinary production of acetone bodies occurred at the sixth hour (second three-hour collection) following the test meal in both the 2nd and 3rd experimental days, showing simply that the greatest *elimination* came between the third and sixth hour. If the greatest *production* in the tissues can be told from the blood figures for "total acetone," that process must have been most active (on the 3rd day at least) in the first hour-and-a-half period. It was not accompanied by a low R.Q. Had there occurred such a coincidence, we might have had reason to regret that bloods were not taken at this time on the 1st and 2nd days when the R.Q.'s were lowest. In short, we again see in these results no near relationship between low quotients and ketosis. Possibly the respiration periods were quite too short for such a purpose. Further discussion of the evidence will follow.

*Adaptation to high fat.*—Except for the more normal quotients on the second, and still more on the third experimental day, there is in this experiment with subject M no evidence of adaptation in so short a time. Perhaps, however, normal quotients constitute as significant a sign as any, and the term should be broadened to include better oxidation (Chart 2).

With subject H also there was *in successive days* on the same diet (not shown in tables) no evidence of adaptation in the ketosis figures; rather

the contrary. Such adaptation as is noticeable, when similar experimental days are laid side by side, occurred many weeks after the original observation. A time element seems to be necessary. The only subject who manifested any sign of adaptation or improved tolerance, so far as the ketosis was concerned, within a few days was subject J, and in his case the change was no greater than the reverse sign, i.e., loss of tolerance within the same time, for subject H. Both, therefore, are probably only day to day variations. It was not possible, because of the rigorous character of the diet, to prolong the observations at a given level sufficiently to determine the minimal time within which the human subject would acquire a definitely greater tolerance. What we are certain of is that the change was apparent in subject H three months after the first trial, i.e., from January to April, on the low-ratio diet and four months (February to May) on the high-ratio diet. Meantime a considerable change of season had occurred, and, as Cori and Cori (11) have shown for the rat, season plays an important part in determining the completeness of fat metabolism. In the fasting rat, however, ketonuria was much accentuated in the summer months



while in the present instance both ketonuria and ketosis were diminished in the spring as compared with the winter. The explanation does not appear to lie in a change of external factors, but of internal.

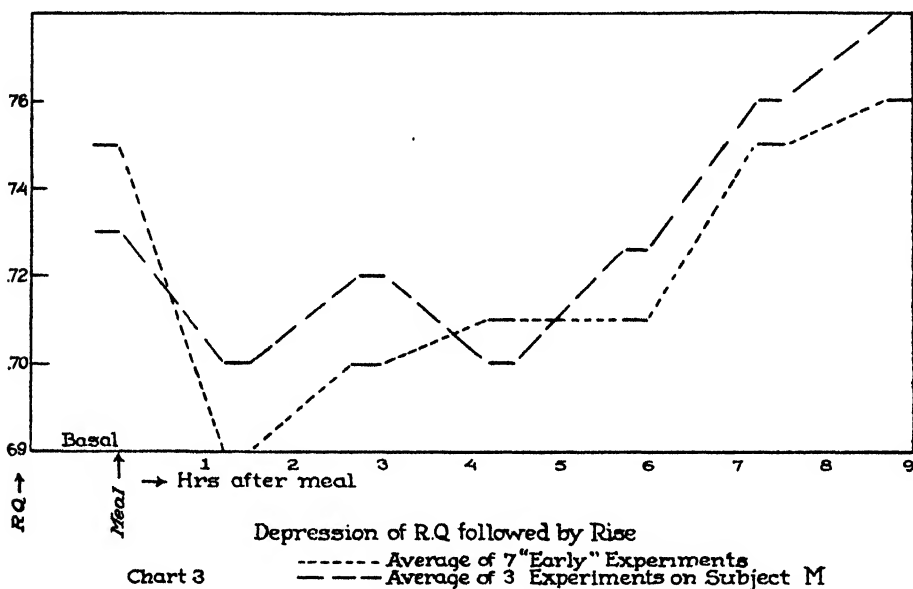
*Effect of low environmental temperature.*—On the hypothesis that the low respiratory quotients soon after a meal of high fat were due to the need for glycogen, any other means of producing the same need at a time when much fatty acid is still available, should induce the same response. Shivering is known to be a good means of removing glycogen. Two of the subjects therefore (V.S. and W., chart 2) were placed in the refrigerator which had been used by Dr. Swift (11a, 12) for his experiments on "The Effects of Low Environmental Temperature upon Metabolism" to see whether shivering would cause a lowering of the R.Q. The refrigerator period came at a time (4–5 hours after the test meal) when the R.Q. usually is rising. The effect is quite pronounced with both subjects. Subject V.S., who was a very thin person, shivered violently for 80 minutes of his sojourn. Subject W, who was quite stout, shivered very little. Accordingly, V.S. showed a greater effect on the R.Q. and this persisted, as shown by the direct air analysis, for some time after his return to ordinary room temperature. If shivering produced the need for glycogen, the low R.Q.'s would seem to prove that any sugar formed was not all burned at once.<sup>3</sup>

### DISCUSSION

The hypothesis with which this work was undertaken was predicated on two demonstrated facts. One, that at best it is difficult for the mammal to convert fat to carbohydrate, and two, that the demand for glycogen as tissue reserve at times dominates the demand for sugar as fuel. Both are abundantly proved. Hence to exhibit conversion it would be necessary to establish rather special conditions; namely, 1.—just that special degree of tissue hunger for glycogen, and 2.—no other adequate source of sugar available, and 3.—the subject, at the moment of test already accustomed to digest and absorb large amounts of fat. With these conditions rightly adjusted, the hypothesis was that a certain sequence of respiratory quotients might be obtained which could not plausibly be explained in any other way. First would come, following a heavy meal of fat, an abrupt drop of the quotient, succeeded after a certain length of time by a rise which

<sup>3</sup> There is, however, a question regarding the effect of low environmental temperature on dissociation of CO<sub>2</sub> from its compounds in the tissues which should receive attention before this conclusion can be made final. The superficial tissues certainly would be affected by the low air temperature (about 3°C). Note that the Benedict method could not be used in the refrigerator.

would go at least as high as the original basals. Averaged for the day the quotients should stand near the expected level for combustion of the ingested fat. Gregg (13) in this laboratory calculated the theoretical quotient for butter fat to be 0.72, instead of the usually accepted 0.707 for mixed food or body fats, the higher level being explained by the presence of lower fatty acids in butter.



The first question to be discussed is, to what extent was the predicted sequence of respiratory quotients realized? Chart 3 shows that as the average result of seven "early" experiments there was a marked depression below the basal level, and that this was followed by a recovery to a level higher than basals. The same chart shows that the average of three successive days with subject M gave a smaller depression but a still higher recovery following. Chart 1 contains several sequences for subject J which exhibit a fall from basal with no, or at most only a transitory, recovery and for subject H, several which ran continuously low throughout the day. Charts 2 and 4 contain some of the most satisfactory (to the hypothesis) sequences in individual experiments, i.e., quotients falling far below theoretical for fat combustion soon after the test meal and recovering above this level late in the day. Such a sequence cannot possibly be accounted for by the secretion of HCl by the stomach, producing a relative alkalosis in the blood and a subsequent reversal as absorption of the chlorine ions

occurs; for in the first place a fat meat produces relatively little gastric juice, second, neutralization in the alimentary tract where very little protein is present to bind up the acid, must take place very promptly after evacuation into the intestine, and, in the third place, the  $\text{CO}_2$ -combining power of the blood always indicates acidosis, if any change, at the point where the quotients are lowest (several other determinations than those given in Tables I, II, and V confirm this). The low quotients are for this reason especially significant for the hypothesis.

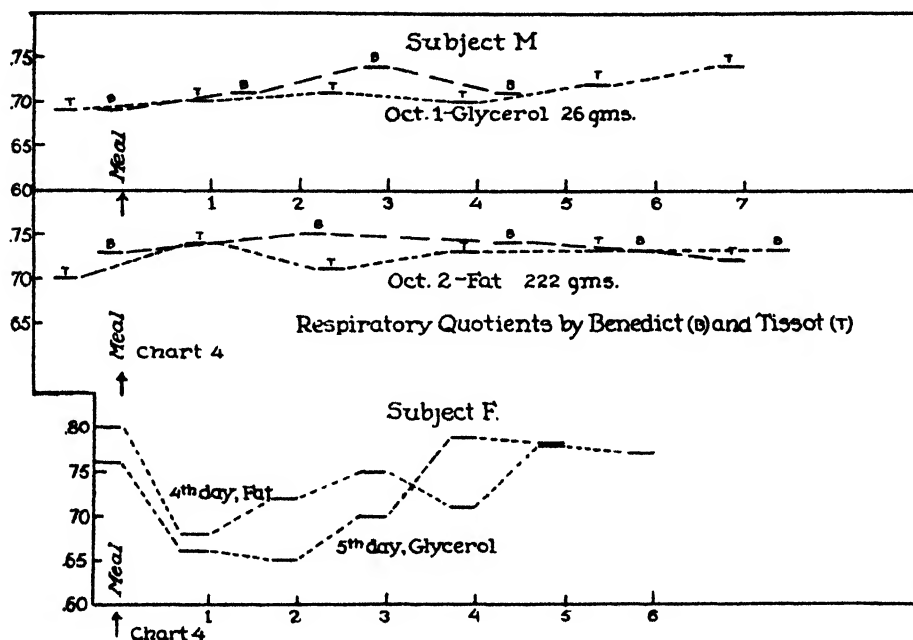
The diversity of results was only to be expected; for unless different subjects evacuated the meal into the intestine quite uniformly one could not expect the sequences of quotients<sup>4</sup> to be closely similar; and even then, they could only be expected to duplicate each other in case the relative demand for glycogen, to the rate of digestion and absorption of fat, were comparable. Disappointingly enough, the most carefully and systematically planned experiments were the least successful from the standpoint of the predicted sequence. These were those upon subjects H and J who took successively the different ratio diets in the hope of hitting upon a correct size of meal, in relation to length of time on the diet to produce a "good" sequence. These conditions could then be applied to other subjects. The phenomenon of adaptation, already discussed, was one development which frustrated this hope. Widely different natural tolerances in the two subjects, extreme alimentary discomfort at times, depressing effects of the ketosis causing occasional loss of periods, alternating periods of rest and great activity, and, finally, probably too much food (calories), were others.

The three subjects, M, V.S., and F, who gave the most "satisfactory" sequences each had been on the all-cream diet only a short time. V.S. had fasted a full day and then had taken nothing but 4X cream for three days. Subject M showed a larger depression of the quotient on the third day of the diet, than on the fourth and fifth. Indeed, as noted on page 534 the general level of the quotients followed the order of the days themselves, indicating acquired capacity to oxidize the fat as such, without any visible improvement from the standpoint of ketosis. This statement sounds contradictory, but will be cleared up presently in discussion of the significance of low quotients in special relation to ketosis.

Concluding the point under discussion at present, it should be stated that while there is a certain gratification in having demonstrated that the

<sup>4</sup> Many of the irregularities in the R.Q.'s shown in Tables I to IV are believed to be due to irregular evacuations of the fat from the stomach into the intestine, the subjects being alternatively quiet and active.

predicted sequence can be obtained, the authors do not consider that low quotients occurring in other than this particular sequence are without significance. Indeed, if we could know the exact order of evacuation from the stomach, the glycogen status of the liver and the food mixtures of the portal vein from period to period, a fortuitously occurring low quotient might turn out to be even more significant than an orderly succession of them followed by high ones.



The next question to be discussed, naturally, is, can these quotients be explained in any other way than by conversion of fatty acid to carbohydrate?

The opinion in this country has come to prevail, mainly through the teaching of Professor Lusk and his pupils, of whom the present senior author has been a convinced believer in this doctrine, that quotients below 0.69 are not trustworthy either in normal or diabetic subjects and can only be accounted for in the diabetic by extreme ketosis or by maximum conversion of protein to sugar. Everybody will admit that quotients below 0.69 are very exceptional and appear only under unusual circumstances of metabolism. Until this work was begun the senior author had never seen such quotients in adult human subjects except when there was good reason to believe that the technic was faulty. From personal participation

in the experiments here reported, and others to be reported later, he is now convinced that they are not only possible but in some subjects they occur quite regularly on high-fat diets.

Looking into the literature with the present hypothesis in mind, the conviction has grown that many such quotients, hitherto regarded as unreliable, probably are reliable. They have been obtained by reliable methods and frequently under conditions not greatly dissimilar to those with which this paper is concerned. A few citations must suffice and those will be confined to researches of the modern period, wherein methods still currently in use were employed.

The celebrated work of Lehmann, Müller, Munk, Senator, and Zuntz (14) on the fasting subjects Cetti and Breithaupt is replete with quotients ranging from 0.65 to 0.68. The method was that of Geppert and Zuntz which certainly is capable of giving correct results when properly used. The authors recognize that such respiratory quotients "appear to be possible only if protein and fat are not destroyed in the normal fashion." Some oxygen rich residue "must be deposited or excreted." The latter possibility is ruled out because the feces of these subjects were normal and the urine showed no such substances in adequate quantity. They calculate that if an average quotient of 0.687 prevailed for a whole day Cetti would have stored some 30.4 gm. oxygen and in a ten-day period there would be 300 gm. oxygen stored in the form of some compound which, proportionally, in experimental animals at least, could not escape detection. They conclude that the storage of oxygen is only temporary, occurring in muscular repose and used up during the balance of the day by muscular work. With the subject Breithaupt quotients of 0.63, 0.66, and 0.69 were obtained in duplicate experiments on the last three days of his 7-day fast and, immediately after, during work the quotients were 0.79, 0.77, and 0.74. The substance which fits these requirements obviously is glycogen and they believe it is formed in repose from body protein.<sup>5</sup>

Bernstein and Falta (15) had as a normal subject a man who for two days previous to their feeding experiment lived on a diet of vegetables and did heavy muscular work for the purpose of removing glycogen from the body. Then for three days on a limited diet containing only 300 gm. meat, 3 eggs and 50 gms. cheese he gave (by the Zuntz-Geppert method) R.Q.'s of 0.66, 0.668, and 0.684. They state that such quotients can only be explained by the formation and excretion of sugar or ketone bodies. No

<sup>5</sup> The authors do not attempt to justify this belief by calculation. Obviously, too, modern methods would require recovery allowances after muscular work,

sugar was present in the urine but a strong ferric chloride reaction was present and levo-rotation. The authors overlook the possibility of temporary storage of glycogen.

To Magnus-Levy (16) is due the credit of first stating clearly the limits of the relationship between oxygen intake and carbon dioxide elimination which are reached by maximal ketonuria in the diabetic. He originated the calculation which was later elaborated and made familiar to American readers by Lusk (17, 18). He states that for the diabetic to attain an R.Q. of 0.65 sugar must come from fat and an R.Q. of 0.60 could only be accounted for if in the metabolism of 200 gms. protein and 250 gms. fat, 300 gms. sugar were formed besides 40 gms.  $\beta$ -hydroxybutyric acid!

Andererseits sei darauf hingewiesen, dass unter Umständen Respirationsversuche eine Bildung von Zucker aus Fett mit Sicherheit beweisen könnten . . . Freilich würden dazu nicht kurze Respirationsversuche ausreichen, sondern mindestens einen, am besten aber mehrere Tage, erstrecken.

Of course such conditions are impossible, and while the interpretation of short experiments is difficult, there is always the satisfaction of reproducibility under given conditions and the conviction that constant results must have definite causes. Much confidence in short-period experiments has been gained in 30 years, because technics are better understood as well as the errors which must be avoided.

Shaffer (19) has well stated that the general principles (which may be expected to produce low quotients) are the same in the normal and the diabetic, and Joslin (20) recognized ten years ago that these low quotients had been found by reliable technics far too often to be longer ignored. Among 113 patients (diabetes) studied by the Benedict "Universal" method between 1908 and 1917, R.Q.'s of 0.69 and below were observed in Joslin's work on 18 occasions with 9 patients in the post-absorptive state. Following a meal of C.35, P.10, and F.55, one patient gave successive quotients on the same day of 0.67, 0.65, 0.67, and 0.58! The next day and the next the same subject gave R.Q.'s as low as 0.62. A number of others showed individual quotients after food (the author does not state at what interval after food in any case!) of 0.67 and 0.68 and several had quotients of 0.68, 0.69, and 0.70 as an average of several periods in which "were included quotients higher than 0.71." Many others even more strikingly unusual could be cited from this work. Ketosis was not measured quantitatively, but the author finds that "acidosis was always accompanied by a falling respiratory quotient." With reference to glycosuria it was notable that amounts of sugar in the 24-hour urine of from 6 to 25



grams were necessary to produce any lowering effect on the R.Q.; amounts between 26 and 100 grams had very little additional effect; but "amounts over 100 grams imply that large quantities of fats are burning, accompanied usually by acidosis, and then the quotient is lowered further." In general a low R.Q. was accompanied by a high percentage of blood sugar and a high R.Q. by a significantly lower, though not necessarily normal, percentage. No direct relationship between blood fat and the R.Q. could be detected.

Wilder, Boothby, and Beeler (21) confirmed the occurrence of low quotients in a single diabetic patient studied over a long period by the Tissot-Haldane method. They found many very low post-absorptive quotients early in the morning—as low as 0.65 (non-protein) and often with very little sugar in the urine and very slight acidosis. Their table on p. 328 gives 0.65 as the average of 8 R.Q.'s following a meal of 85.5 grams fat. Table II on p. 331 shows several tests of the specific dynamic action of high fat meals of which the following are illustrative.

Basal R.Q.'s	June 1 0.69	June 2 0.68
Breakfast containing 34.9 gm. protein and 42.1 gm. fat		
After meal		
½ hr.	0.61	0.62
1 hr.	0.63	0.65
2 hrs.	0.65	0.65
3 hrs.	0.64	0.66

The authors state that they are certain these quotients were correctly obtained, but that "it is impossible, as Professor Lusk has emphasized, to make correct allowances for such factors as temporary alteration in the character of the respirations, so common in all respiration experiments, for variation in the CO<sub>2</sub>-combining power of the blood, depending on whether acidosis is increasing or diminishing at the time of the test, and for utilization of oxygen and carbon dioxide in the formation of acetone bodies." They state also that this patient "tried so hard to be quiet and breathe normally that at times during the collection of expired air there was under-ventilation of the lungs, causing retention of CO<sub>2</sub>." We take no exception to the statement that it is difficult to make proper allowances for disturbing factors. Nevertheless, it is necessary to make the attempt, particularly as regards the influence of acidosis. That is one of the major objects of

the present study. Those influences have been overestimated, as will presently be shown. As for underventilation of the lungs, it strikes the present writers as exceedingly unlikely that underventilation should recur at collection periods so regularly and in just the proper variation of degree to produce that particular sequence of quotients which, according to the present hypothesis, would be produced by formation of oxygen-rich material from oxygen-poor, and the retention and subsequent oxidation, or partial oxidation, thereof. It is one of the advantages of the Benedict method, carried out with a recording spirometer, that one can be sure of the normal depth of respiratory movements.

Neither Joslin nor Wilder, Boothby, and Beeler attempted a theoretical discussion of the limits of their low quotients in relation to the characteristics of metabolism. That service was rendered by Shaffer (19) in one of his discussions of anti-ketogenesis. He found that a total R.Q. of 0.76 indicated the oxidation of a metabolic mixture made up approximately of equimolecular amounts of ketogenic substances (fatty acids or ketogenic amino acids) and of antiketogenic derivatives of amino acids, glycerol, or carbohydrate, expressed as total glucose. A quotient of 0.73 indicated 2 mols. of ketogenic to 1 of antiketogenic substance, while one of 0.80 indicated 0.5 mol. of keto to 1 of antiketogenic glucose equivalent. Later Shaffer (22) modified the rigidity of these proportions by admitting that some subjects undoubtedly show small but definitely abnormal amounts of acetone when mixtures of metabolites, judged by the R.Q.'s or by calculation, correspond to a ketogenic:total glucose ratio of about 1:1; while other subjects may have a ratio of about 2:1 before large amounts of acetone bodies appear in the urine.

Baer (23) long ago noted that different mammalian species react differently to withdrawal of carbohydrate. While man and monkey react with acidosis on mere withdrawal, the swine exhibits it only in complete starvation, the dog, goat, and rabbit only when poisoned with phlorhizin.

Lyon, Dunlop, and Stewart (24) recently have found many low quotients in obese patients taken in the post-absorptive condition. Out of 15 determinations on subjects whose reducing diet was low in carbohydrate and contained approximately 1000 calories the quotient was below 0.70 in 12, or 80 per cent, of the tests. Such quotients occurred on higher caloric intakes, but much less frequently. Every possible precaution seems to have been taken. Their computation of maximum possible yield of carbohydrate from protein and glycerol confirms our own, made before their contribution appeared (see p. 549).

TABLE VI  
AVERAGE ACETONE PRODUCTION ON DIFFERENT FAT LEVELS—SUBJECT J (MALE)

Total Urinary Acetone, Mgms./hr.					
Date 1930	F.A.:G Ratio	Basal	3 hrs.	6 hrs.	9 hrs.
Jan.	1.5:1	Negative to ferric chloride			
Jan.	3.0:1	25	57	70	24
Feb.	4.1:1	40	63	61	45

Total Blood Acetone Mgms./100 cc.					
Jan.	1.5:1	9	7	8	—
Jan.	3.0:1	16	13	14	19
Feb.	4.1:1	24	40	40	32

ADAPTATION TO FAT—SUBJECT H (FEMALE)

Urine—Total Acetone, Mgm./hr.					
Jan.	1.5:1	11	—	—	—
Jan.	3.0:1	171	274	353	364
Feb.	4.1:1	324	—	—	350
Apr.	1.5:1	18	18	20	—
May	4.1:1	187	216	206	—

Blood—Total Acetone, Mgm./100 cc.					
Jan.	1.5:1	42	—	40	—
Jan.	3.0:1	55	52	51	65
Feb.	4.1:1	58	43	51	41
Apr.	1.5:1	14	8	14	—
May	4.1:1	29	39	27	25

No established fact in the literature, it is believed, directly contradicts the hypothesis that low respiratory quotients reliably obtained under the conditions described on page 524 may denote some conversion of fat to carbohydrate. Periods of activity alternating with periods of repose in fasting, as in Lehmann and Zuntz's work, depletion of glycogen followed by a meal containing little or no carbohydrate but much fat in normals as in Bernstein and Falta's experiments, heavy fat feeding to diabetics as in Joslin's and Wilder, Boothby, and Beeler's studies, low carbohydrate, low calorie diets for the obese, have demonstrated many low quotients. We believe systematic attention to the conditions we have described as essential would demonstrate many more. The effects of acidosis will not furnish an

answer to the question of the *possibility* of gluconeogenesis from fat as will be seen below.

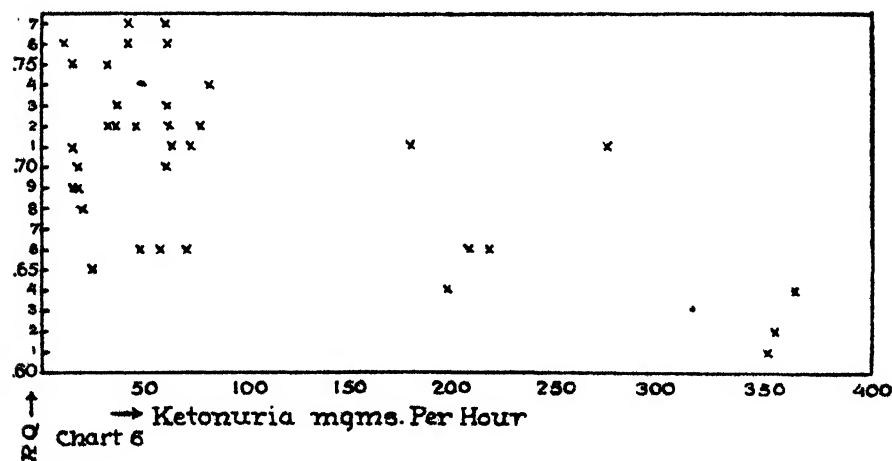
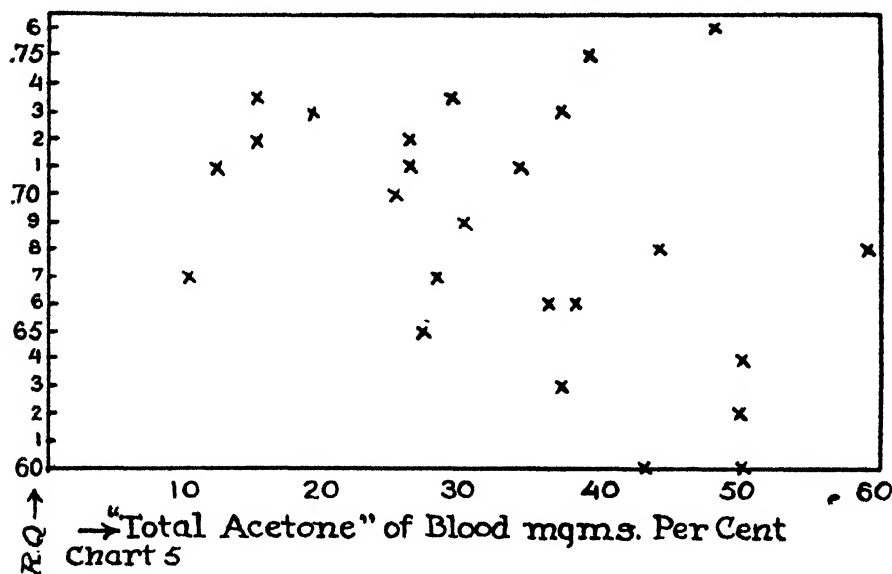
*Relation of actual R.Q.'s to ketosis.*—That the degree of acidosis bears a direct relationship to the amount of fat fed was to be expected, and, with allowance made for the phenomenon of adaptation, is perfectly evident in these experiments, as may be seen from Table VI which summarizes results for subjects J and H. Respiratory quotients,<sup>6</sup> however, were not found to depend on the amount of fat in the diet but rather upon the condition of the subject at the time the fat was taken. A person with a very high natural tolerance or a recently acquired tolerance for high fat was apparently not so good a subject for producing low R.Q.'s as a person with low tolerance.

Chart 5 exhibits the wide scatter which one obtains in attempting to show a relationship between R.Q.'s and the ketosis as represented by "total acetone" of the blood. Three subjects, H, J, and M furnish the data, but not at all equally. Some of the "points" represent averages of as many as six different amounts of total acetone for a single R.Q. Plotting all the individual points only increases the scatter. The chart therefore represents the most conservative, legitimate use of the data. When a similar chart containing different symbols for each of the three subjects was prepared, there was no grouping characteristic of each subject. Examination of Tables I to V has already confirmed this impression.

Chart 6 exhibits an even wider scatter concerning the relationship between the ketonuria and R.Q. In this instance, however, there is a tendency toward an individual grouping. All of the "points" above 150 mgm. "total acetone" per hour were contributed by subject H, but by no means all of the points contributed by this subject lie in this range. Of all the points below 100 mgm. 11 were contributed by subject J, 12 by subject M, and 4 by subject H. The quotients corresponding to these points range from 0.65 to 0.77. The chart gives a very faint impression that a much larger collection of data would exhibit a broad drift of coördinate points from the upper left to the lower right hand corner of the field. Even so, the scatter would be quite too wide to denote anything like a close correlation. The conclusion from these two charts inevitably is, that the R.Q. is not intimately dependent upon the ketosis, as judged by blood analysis or excretion. The only way in which ketosis could be linked with the quality

<sup>6</sup> In these experiments where the protein metabolism was so small compared with the total, no material difference results whether total R.Q.'s or non protein are used. All quotients unless otherwise specified are total.

of the respiratory metabolism in these experiments would be to suppose that following the test meal there must be a considerable accumulation of ketone bodies in certain tissues before they appear even in the blood.



*Relation of R.Q. to protein metabolism.*—Lehmann and Zuntz surmised that low quotients in their fasting subject might be due to the formation of glycogen from protein. By analogy with the formation of sugar from protein in diabetes, this suggestion strikes one as very plausible. The ques-

tion is, how much of a depression below fasting level could be accounted for in this way. Fortunately we have a time-honored, though not necessarily unobjectionable, method of calculation which may be used to gain an idea of the effect. It is employed here for want of a better. Magnus-Levy (16) originated the method and it has been employed by Loewy, by Frentzel and Schreuer, Geelmuyden, Lusk, and others. Before putting it to a test we must select a period and translate the metabolism as given in calories per hour into grams of fat and protein. The heat production for quotients below 0.707 in previous tables has been calculated as if the quotient were actually at this level. There is as yet no widely accepted table of heat values of oxygen for non-protein quotients below this. Bernstein and Falta (15) prepared by extrapolation from the Zuntz and Schumburgh standards a table of values down to 0.60. This will be used *for our purpose now* with the conviction only that it is the lesser of two fallacies. In a later paper it is hoped to give more exact values from direct calorimetry.

Selecting the period from 3rd to 6th hour after food on April 8 for subject H (Table I) we find that 0.436 gram N per hour was excreted. The heat value of this metabolism would be 11.55 Calories, the non-protein R.Q. works out at 0.65, the calories from fat by Bernstein and Falta's table would be 46.53 Cal. and the grams fat metabolized in the hour 4.897 at a caloric value of 9.5 per gram.

Assuming an availability of 58.5 per cent (D:N, 3.65) the protein metabolized would yield 1.592 grams sugar. This amount of sugar, however, would make only 1.434 gm. glycogen. The effect of this glycogen formation and the substitution of an isocaloric quantity of fat in combustion, may be shown in the conventional manner as follows:

	C	H	O
1 gm. N-free protein contains,	0.415 gm.	0.044 gm.	0.0769 gm.
2.725 gm. N-free protein (N×6.25) contains,	1.130	0.1199	0.2092
1.434 gm. glycogen contains	0.637	0.0889	0.7084
	<hr/>	<hr/>	<hr/>
Leaving	0.493	0.0310	-0.4992
	<hr/>	<hr/>	<hr/>
0.638 gm. fat, isocaloric for glycogen,	0.488	0.0759	0.0733
	<hr/>	<hr/>	<hr/>
Making a total of	0.918	0.1069	-0.4159
Adding for 4.897 gm. fat (see above)	3.746	0.5828	0.5632
	<hr/>	<hr/>	<hr/>
Total	4.727	0.6897	0.1473

Deducting for intramolecular $H_2O$		0.0184	0.1473
Remaining to be oxidized,	4.727	0.6713	0.0
Requiring 17.943 gm. $O_2$ and producing 17.301 gm. $CO_2$			
In liters, 12.550 L. $O_2$ and 8.823 L. $CO_2$			
R. Q. = 0.703			

The R.Q. for the protein and fat metabolism as calculated from the actual data by the same sort of procedure as above, but omitting the data for glycogen and the substituted fat, would be as follows:

	C	H	O
1 gm. N-free protein contains	0.415 gm.	0.044 gm.	0.0769 gm.
2.725 gm. N-free protein contains	1.130	0.1199	0.2092
4.897 gm. fat protein contains	3.746	0.5828	0.5623
Total	4.876	0.7027	0.7715
Deducting intramolecular $H_2O$ ,		0.0957	0.7715
Remaining to be oxidized,	4.876	0.607	0.0
Requiring 17.826 gm. $O_2$ , producing 17.846 gm. $CO_2$			
In liters 12.474 L. $O_2$ producing 9.086 L. $CO_2$			
R.Q. = 0.728			

The effect of the formation of glycogen and the substitution of an isocaloric quantity of fat in combustion would be to reduce the R.Q. from 0.728 to 0.703, a depression of 0.025. This particular period represents the greatest ratio of protein metabolism to the non-protein metabolism observed for subject H. The correction therefore is maximal.

The period which revealed the lowest protein metabolism in relation to the non-protein for this subject is the last period of February 2 (Table III) where the N output per hour was 0.163 gm. The same calculation for this period shows that formation of glycogen and substitution of fat isocalorically for the fuel thus lost would depress the R.Q. exactly .01. The correction from this cause may therefore be placed at not less than .01 nor more than .025. Examination of the data for the other subjects for whom the protein metabolism was known has convinced us that the range of correction for them would come within the same limits. In the particular periods chosen for illustration the correction for the larger protein metabolism would raise the observed R.Q. from 0.68 to 0.705; for the lower protein metabolism it would raise the observed R.Q. from 0.61 to 0.62. It is obvious that glycogen formation from protein cannot account for the lowest quotients obtained with subject H, but that it might account

for a slight depression. Obviously, too, the combustion later of glycogen from this source would account for a rise of quotient above the theoretical. (See also the computation of Lyon, Dunlop, and Stewart.)

Meantime there is to be considered a combined correction for ketosis and alteration of protein metabolism occasioned thereby.

*Correction for ketosis and ammonia formation.*—There is no reason from the data of this paper, or from the literature, to suppose that a correction to the R.Q. for the degree of ketosis found would be significant. Nevertheless to make all the corrections possible it has been carried through. The general method is familiar and can be found in Lusk's "Science of Nutrition," 4th edition, p. 671. In the present instance the correction has been made on the basis of the increase of the acetone bodies in the blood during the three-hour period within which the respiration period lies. Acetone and diacetic acid have been treated as one quantity,  $\beta$ -hydroxybutyric acid as another. In order to make the correction very generous the quantities in the blood have been assumed as applying to the *entire weight of the body*. The result is to raise the non-protein R.Q. by an amount from .007 to .02 in the three instances shown in Table VII. Actually the increase would not be more than 1/5 of this amount, even allowing for some formed ketones which have not appeared in the blood! Magnus-Levy calculated that a formation of 40 grams  $\beta$ -hydroxybutyric acid would lower the R.Q. only .012; correction for this large formation therefore would raise it only by a like amount.

Against this stands the correction for production and excretion of ammonia instead of urea, used in neutralizing the ketonuria. In Table VII also are found the changes in non-protein R.Q. occasioned by correction for the diminished oxygen absorption caused by shunting off  $\text{NH}_3$  before it is changed to urea. The R.Q. is higher because of ammonia excretion; the correction therefore lowers it by the same amount. For the largest amount of ammonia shown (subject H for Feb. 2) the correction is .01 downward. For the least amount shown it is negligible. The net change for both corrections is found by comparing the first non-protein R.Q. in Table VII with the last.

*Conversion of glycerol to sugar.*—It is generally agreed that in diabetes sugar can be formed from glycerol. The simplest equation is,  $2\text{C}_3\text{H}_8\text{O}_3 + \text{O} = \text{C}_6\text{H}_{12}\text{O}_6 + \text{H}_2\text{O}$ , i.e., dehydrogenation. Should the same take place in the normal subject what effect would be produced on the R.Q? The attempt to answer this question has been made experimentally. Glycerol in amount equivalent to the theoretical total yield of the fat ingested on an adjacent



day was given to two different subjects. They reacted quite differently, as may be seen from Chart 4. Subject M was studied by both the Benedict and the Tissot-Haldane methods on October 1 when glycerol was taken and on October 2 when the fat was ingested. Previous to the glycerol day were 3 days of high-fat (all cream) diet. With only one exception on each day the R.Q.'s taken one-half hour apart by the two methods, agreed remarkably well. Comparing the Tissot results on the two days because they are more complete, they are, with the exception of the first hour period as nearly duplicate results as one could ever hope to get on two successive days following identical meals. There was, however, no early drop in the R.Q. because, on the present hypothesis, there was no demand for glycogen at this time. Why there was no demand may be explained by the phenomenon of adaptation. Subject M had been through the high-fat experiment in May preceding and at that time exhibited some signs of adaptation (See Table V) to the diet, not connected with ketosis. As with subject H, on resuming the diet several months later better tolerance was shown. It is useless at present to speculate on just what all is implied in better tolerance. The manifestation of it for present interest is the nearly uniform combustion qualitatively throughout the experimental day. It is astonishing that with a meal furnishing so little energy as did the glycerol, as compared with the fat meal, the metabolism could be so nearly the same qualitatively. Quantitatively the two days were quite dissimilar. Instead of a large dynamic effect which is obtained regularly from the cream diet, there was on the glycerol diet an actual depression of heat production which continued for many hours. But that difference need not detain us at present.

Subject F was studied by the Tissot method only. After three days of the high-fat (ratio 4.1:1) diet the subject took a test meal consisting of 665 grams 4X cream, containing approximately 249 grams fat, and the respiratory metabolism was followed for six hours thereafter in hourly periods. On the fifth day the meal consisted of 26.6 grams glycerol. The R.Q. curves are shown in Chart 4. The agreement is not quite so good as on the two days for subject M, but the early fall and subsequent rise in the quotients are striking and the average of the first four periods, following the first low quotient, is the same, 0.74, on the two days, showing that the recovery was on the average the same, so far as they could be compared.

Both experiments are strikingly suggestive of an identity of effect from glycerol as such and from glycerol included in the fat. It would seem that we have in the conversion of glycerol to sugar (glycogen) an adequate

TABLE VII  
CORRECTION OF NON-PROTEIN R.Q. FOR  $\text{NH}_3$  AND ACETONE BODIES

Subject	Urine N	Non-prot. R.Q.	Urine $\text{NH}_4\text{N}$	Corrected non-prot. R.Q. for $\text{NH}_4\text{N}$	Acetone + diacetic gm.	$\beta$ -hydroxy- butyric gm.	Gms. blood incr. or decr.		Corr. non-prot. R.Q. for ketosis
							Acetone diacetic	+ $\beta$ -hydroxy- butyric	
H	0.163	0.70	0.049	0.69	0.72	0.278	0.0	+0.2	0.702
J	0.222	0.67	0.018	0.67	0.011	0.047	0.33	+4.35	0.69
M	0.372	0.724	0.036	0.722	0.012	0.081	+7.0	+1.66	0.729

explanation for the low quotients. In the experiment on subject F, where glycogen was needed, and in subject M where it was not needed, the glycerol was metabolized with body fat just as it was with the cream fat.

The effect on the R.Q. of the conversion of glycerol to glucose may be pictured with substantial accuracy as follows. Arguing that the glycerol given as such to subject F was completely converted to sugar within 3 hours and that 2/5 of the transformation occupied the first hour, 2/5 the second and 1/5 the third,<sup>7</sup> there would be 10.64 (2/5 of 26.6) grams available the first hour. The requirement for oxygen would be  $(10.64 \times 16/184) = 0.925$  gms. or 0.647 liter. Subtracted from the oxygen absorbed that hour (20.6 liters) this would leave 19.95 liters which, divided into the  $\text{CO}_2$  production (13.6 liters), would give a quotient of 0.682 instead of 0.660, a difference of .022. Probably this is a maximal correction from this cause. Assuming conversion at the same rate on the high fat day, the correction would be only .015 because of the much greater oxygen absorption occasioned by the specific dynamic action. The sequence of the R.Q.'s on this day indicates that the conversion did not proceed quite so rapidly or so far as on the glycerol day. In any event it is clear that the conversion of glycerol to sugar (glycogen) would need to proceed even more rapidly than estimated above to account for the sagging of the quotient within the first hour after absorption. *One has difficulty also in picturing how glycerol can be split off for glycogen formation faster than fat is burned;* for so long as the fat is in transport or in deposit the glycerol is required to keep it neutral. The largest

<sup>7</sup> These fractions are based on the increases in oxygen absorption.

hourly combustion recorded in this study is 115.5 Cal. After deducting for protein metabolism, the balance is accounted for by combustion of 10 grams fat. Ten per cent of this would be glycerol. The effect of the conversion of 1 gm. of glycerol to glucose would be negligible in such a total combustion.

*Combined corrections.*—Should conditions ever arise in which glycogen would be demanded from the protein metabolism and the glycerol of the fat metabolism simultaneously, this combined conversion at maximal intensity would not account for a depression of more than .03 in any hourly period of this study. Adding the largest actual net correction imaginable from the two phases of ketosis, the total effect could not be more than .04. If the normal R.Q. for the all-cream diet be taken at 0.73,<sup>8</sup> then any R.Q. below 0.69 in these experiments calls for some other explanation. There are far too many of that class to be charged to errors of technic or undetectable alterations of respiratory movements. They have been obtained by both Tissot-Haldane and Benedict methods. On the same day (February 2) within one-half hour of each other by identical technic on the same Benedict machine, one subject (H) gave an R.Q. of 0.60 and the other (J) 0.75. Subject H gave by far the largest number of low R.Q.'s and there was at the same time plenty of evidence that she could not properly burn the fat—low specific dynamic action, marked ketosis, etc. Make all the corrections possible for glycogen formation and ketosis (they rarely go together) and the R.Q.'s still are far below normal for combustion of fat.

*Other possible use for oxygen.*—It is not necessary to conclude hastily that fatty acids go to sugar as readily as sugar goes to fatty acids, as many young authors, trained to regard all reactions as reversible, are inclined to do. If that were true it would not be possible to render dogs and cats glycogen-free on a cream diet, as Dale's laboratory<sup>9</sup> claims to have done! There is one other possible use for excess oxygen which we have not seen mentioned in the literature. On the modern theory of beta-oxidation of fats there is a clear possibility that a considerable amount of oxygen may be employed in the formation of intermediary acid radicals and other groupings before any carbon dioxide production gets well started. With an enormous flood of fat entering the circulation, it would seem that intermediary partially oxidized bodies not yet small enough to be water-soluble and excretable by the kidney would account for enough oxygen to lower

<sup>8</sup> This would be a minimal average, allowing for protein metabolism and a technical error of .02.

<sup>9</sup> See paper by Gregg, D. E., *Amer. Jour. Physiol.*, 1933, 103, 79.

the R.Q. for several hours, possibly all day. Later, perhaps when the anti-ketogenic effect of glycerol in larger amount becomes available the oxidation quickly proceeds to the end and there is a larger elimination of  $\text{CO}_2$ . Several sequences in the tables suggest this. The initial drop in R.Q. is due more often to a rise of  $\text{O}_2$  than to a change in  $\text{CO}_2$ ; later when the R.Q. rises, not only is the oxygen falling but the  $\text{CO}_2$  is rising. In this view of the matter it would not be the glycogen hunger alone but the lack of catalysts (oxidases?) perhaps which would establish the condition precedent for low quotients following high fat.

The conception here advanced is not unlike that of Geelmuyden (25) in that the intermediary substances would contain more oxygen (or less hydrogen); but differs from Geelmuyden's ideas in that the desaturation would not need to proceed to the carbohydrate stage to account for the quotients alone. The theory here advanced will be discussed in a later paper.

The authors do not claim to have proved the conversion of fat to carbohydrate in the human body. They think the low quotients are a reality; they feel certain that neither glycogen formation from protein and glycerol nor ketosis, nor both can, in these experiments, account for quotients lower, on the average, than 0.69. If it could be shown that no other use of extra oxygen were possible, or if the sugar supposed to be formed from fat involving these low quotients could be found, the proof in either case would be logical and complete. It is the authors' conviction that the extra sugar from fat never has been clearly demonstrated. Logic compels us to wait for further evidence.

#### SUMMARY AND CONCLUSIONS

1. Respiratory metabolism studies on seven different subjects taking high-fat diets, following meals containing varying amounts of butter fat, show many respiratory quotients below the theoretical level for oxidation of fat.

2. The occurrence of these low quotients does not depend upon the amount of fat taken in the experimental meal, nor upon the F.A.:G ratio of the general diet, so much as upon the tolerance of the subject.

3. Adaptation to or tolerance of high fat in the sense of better capacity to oxidize fat and producing less ketosis may be acquired and be retained for several months.

4. The level of the respiratory quotient bears no intimate relationship to the demonstrable ketosis or ketonuria.

5. The hypothesis with which the work was undertaken, calling for a

special sequence of low quotients soon after the test meal followed by higher quotients later, has been realized in a number of individual experiments, but not in all. The most successful from this point of view are the early experiments for each subject performed before increased tolerance had developed. Low quotients may be induced to appear also by shivering at a time when, ordinarily, they would be high.

6. Production of glycogen from the protein metabolism could account for a depression of the R.Q. at most of .025; while production of glycogen from glycerol, assuming that only the glycerol of the fat metabolism were available, would produce a depression of not more than .003. Correction of the quotient for the demonstrable ketosis and consequent ammonia formation would not account for more than .005. At most the combined effect of all these factors would not account for quotients lower than 0.69.

7. The formation of glycogen from fat (beyond the amount which could arise from glycerol) having never been proved, it would be premature to conclude that the quotients below 0.69 in this work demonstrate gluconeogenesis from fatty acids.

8. It is suggested, as an alternative explanation, that in the oxidation of fatty acid chains the uptake of oxygen may considerably outrun for a time the production of carbon dioxide and thus account for a depression of the R.Q. A process of desaturation which would remove hydrogen, but not produce any carbon dioxide, followed by oxidation with production of  $\text{CO}_2$ , would fulfil the requirements.

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## Editorial Review

### OTHER FACTORS THAN VITAMINS B AND G IN THE VITAMIN B COMPLEX\*

OUR knowledge concerning the multiplicity of factors in the vitamin B complex is constantly undergoing revision as a result of an ever-increasing number of contributions to this field of vitamin research. The accumulation of evidence forces the most conservative to conclude that not only one but probably several components exist besides the two better defined factors, the antineuritic B(B<sub>1</sub>) and the antipellagric G(B<sub>2</sub>). Our uncertainty as to the nature of these additional components may be well emphasized by quoting from the recent review of vitamin B by Kruse and McCollum (28). They state, "Of the seven components at present advanced as constituents of the water-soluble B complex, the pigeon needs three, and the rat requires five for optimum nutrition. Thus far only one component, the antineuritic vitamin B, has been shown to be definitely needed in common by the two species; on the other hand, there is some evidence that certain components are required by one species and not by the other. The entire question of the nutritive needs of the pigeon and the rat for growth is in a transitional state, and judgment concerning it may well be reserved. It is not impossible that the estimated number of B components may be reduced by the demonstration that certain of them are identical. However, the available evidence that the pigeon and the rat require different components of the B complex for maximum weight is of such convincing character that it cannot be dismissed."

This newer knowledge concerning the "ultra-complexity" of the vitamin B complex will be far reaching in its effect on vitamin studies. Radical changes must be instituted in the methods used for the study of any single one of the components of the vitamin B complex. Much of the work that has been done in the past on vitamins B and G is subject to criticism on account of failure to insure the presence of all components, other than the one being studied, in the diets employed. Quoting again from the article of Kruse and McCollum (28) on the antineuritic factor, "Certainly there is abundant opportunity for error in attempting to correlate the pathologic and physiologic manifestations of the rat and the pigeon on a defective diet: unless the ration of each species is made adequate with respect

\* The material contained in this review was taken from a paper read before the Biological Division of the American Chemical Society at the Denver meeting in August, 1932, as part of a symposium on the so-called "third" factors of the vitamin B complex.



to all factors required by it, with the exception of the antineuritic component, the observations may not be comparable." What is true of the antineuritic factor B is equally applicable to G or to any other component of the vitamin B complex. It is obvious that in the future more care must be taken in the preparation of basal diets for the study of the better defined factors, B and G, as well as of the newer allegedly existing components.

The present attempt to review the literature on the so-called "third" factors of the vitamin B complex seems particularly appropriate, inasmuch as there are only two such surveys that might be considered in any sense comprehensive. The first by Peters (32) was presented two years ago and only two years after the first paper on the "tripartite" nature of vitamin B appeared; the second by Harris (21) is contained in a review of recent literature on all of the vitamins and, although it contains a valuable list of contributions to the literature on the subject, "exigencies of space have excluded a discussion seriatim of the separate factors." Although both articles are well worth the attention of workers in this field, as well as that of others less directly interested, a comprehensive review of the literature at this time is quite opportune when one realizes that a considerable number of papers have appeared since Peters' review.

The difficulty of any effort to correlate the results of the different workers in this field needs no great emphasis. However, when one considers that the methods employed by different workers vary tremendously, that the evidence is oftentimes presumptive and based upon feeding experiments where sub-normal growth has been observed with diets supposedly adequate in all of the known factors, that very few of the workers have attempted to fractionate the factors from a single source rich in all of the components of the vitamin B complex, and that the evidence of new factors is not based upon the production of characteristic lesions due to the supposedly "new" vitamin deficiency, one approaches a critical analysis of the subject with considerable temerity. Especially is this true when one realizes the validity of the recent statement of Harris (21) that "any direct comparison or collation of results is rendered almost impossible by the fact that widely diverging definitions and basal assumptions are taken by the different investigators." The review of the literature that follows is made with a full realization of these attendant difficulties.

### *Vitamin B<sub>1</sub>*

In 1927 Williams and Waterman (51, 52) reported that pigeons, brought to a subnormal weight level on a synthetic diet free from vitamin B com-

plex or on a diet of polished rice, required for weight restoration some other factor than the antineuritic vitamin B and the antipellagric vitamin G. The basis of their claim was the fact that, although the decline in weight on such deficient diets could be stopped by the addition of a special activated fullers' earth preparation of the antineuritic factor from yeast, restoration of weight failed to occur even when autoclaved yeast as a source of vitamin G was given as a further supplement, but did take place upon the addition of air-dried brewer's yeast. Apparently, the recovery in weight, and the accompanying improvement in general condition, must have resulted from the presence of a thermolabile factor in the yeast other than the antineuritic vitamin B. Whole wheat was also shown to contain this "new" factor. Furthermore, it was demonstrated that rats do not require this second thermolabile component of the vitamin B complex. This factor has been designated vitamin B<sub>2</sub> by Williams and Eddy (50).

Randoin and Lecoq (34) had shown in 1926 that pigeons must receive, in addition to the antineuritic vitamin, a growth-promoting or nutritional factor ("*vitamine d'entretien ou de fonctionnement*" or "*vitamine d'utilisation nutritive*"), and that certain yeasts or yeast extracts prevented loss of weight but not polyneuritis in adult pigeons. In the following year Lecoq (29) claimed that at least three factors in the vitamin B complex, the antineuritic factor, a thermo-stable, alkaline-stable, growth-promoting factor, and the "nutritional factor" of Randoin and Lecoq, are needed by the pigeon. As Randoin and Lecoq had demonstrated that their nutritional factor was destroyed by autoclaving in an alkaline medium, Lecoq (30), in discussing the question of the identity of the nutritional factor of Randoin and Lecoq and the Williams and Waterman vitamin B<sub>2</sub>, expressed the opinion that they are the same.

The requirement of pigeons for the Williams and Waterman vitamin B<sub>2</sub> has been further confirmed by Peters (32) and by Eddy, Gurin, and Keresztesy (12). The latter investigators have shown that this vitamin B factor is also needed for normal growth by chicks, even when fully supplied with vitamins B and G. They have demonstrated that vitamin B<sub>2</sub> is even less stable to heat than vitamin B, especially in alkaline solution, thereby confirming the original report of Williams and Waterman that their factor was exceedingly thermolabile.

#### *Vitamin B<sub>4</sub>*

In 1929, Reader (36) reported evidence for the existence of a third factor in the vitamin B complex. Addition of vitamin B concentrates, prepared according to Kinnersley and Peters (27), and of vitamin G, as al-

kaline autoclaved yeast extract, to a basal diet deficient in the vitamin B complex did not induce normal growth of rats even when the amounts of B and G were increased above the minimum requirement. Growth either ceased or proceeded at a very subnormal rate. The substitution of marmite (whole yeast extract) for the B and G resulted in a resumption of growth to maximum weight. This indicated that yeast contains, in addition to B and G, a third factor necessary for normal growth of the rat. This third component was shown to be very thermolabile, much more so than vitamin B. In a later paper, Reader (37) reported the preparation of a concentrate of this factor from an extract of the mercuric sulphate precipitate formed in the process of Kinnersley and Peters (27) for the preparation of vitamin B. Experiments with this concentrate completely confirmed the earlier work on the existence of a second thermolabile rat factor. Although she had tentatively adopted the term vitamin B<sub>3</sub> for this rat factor, Reader (38) finally suggested that vitamin B<sub>4</sub> be used to denote the third rat B factor, thus avoiding confusion with the Williams and Waterman thermolabile pigeon factor which had been termed vitamin B<sub>3</sub>.

Soon after Reader's first paper, Chick and Roscoe (8) questioned whether the results obtained by Reader were due to a deficiency of a new vitamin, and suggested that they might have been caused by an insufficiency of vitamin G, a portion of which had been destroyed by the alkaline autoclaving used in the preparation of the vitamin B<sub>4</sub> supplement by Reader. Later, however, Roscoe (40), as a result of having observed in negative control rats the listlessness, hunched attitude, and inflamed and swollen paws typical of vitamin B<sub>4</sub> deficiency as described by Reader, admitted the existence of vitamin B<sub>4</sub> and gave evidence for its distribution in vegetables. Halliday, Nunn, and Fisher (20) have recently described a thermolabile "third" factor necessary for the growth of rats that seems to be identical with the vitamin B<sub>4</sub> of Reader.

#### *Vitamin B<sub>5</sub>*

In his Harben lectures, Peters (32) gave evidence that pigeons require another factor of the vitamin B complex in addition to vitamins B and B<sub>3</sub>. Whereas preparations of these two vitamins were not sufficient as supplements to a diet deficient in the vitamin B complex to maintain the weight of pigeons, this was accomplished with fresh marmite (yeast extract) which lacks vitamin B<sub>3</sub> (Williams and Waterman factor). This new B component was shown to be destroyed by alkaline autoclaving, and Peters expressed the belief that it might be identical with the rat factor B<sub>4</sub>.

of Reader. Later, however, Carter, Kinnersley, and Peters (4, 5) concluded that this maintenance factor for pigeons was different from the rat factor  $B_4$  of Reader, since it was heat-stable to an alkaline heating that would destroy vitamins B and  $B_4$ . Furthermore, they advanced other arguments against the identity of their maintenance factor, which they termed vitamin  $B_8$ , and the Williams and Waterman vitamin  $B_8$ .

As mentioned earlier in this paper, Randoin and Lecoq (35) demonstrated the requirement by pigeons of a thermostable, alkaline-stable, growth-promoting factor in yeast. Although they believed that this factor was identical with the antipellagric vitamin G, the fact that this latter vitamin is now generally considered not to be a requirement of pigeons makes it seem probable that they were dealing with a component identical with Peter's vitamin  $B_8$ .

#### *"Factor Y" of Chick and Co-workers*

Chick and Roscoe (7) reported that rats fed a diet in which egg white was the sole source of the vitamin B complex failed to grow even when vitamin B was added as Peter's antineuritic concentrate, whereas rats on autoclaved yeast and B grew normally. As they had demonstrated that egg white was a rich source of vitamin G free from vitamin B, the presence of a thermostable "third" factor in the vitamin B complex was suggested. Further evidence for the presence of this thermostable factor in yeast was given by Roscoe (39) and by Chick and Copping (6). In the latter paper it was shown that yeast extract, autoclaved under conditions sufficient to destroy completely its G content (4 hours; 120–125°; pH. ca. 10) still retained this new factor, which they designated as "Factor Y."

They emphasized the fact that Factor Y differs from the  $B_8$  of Williams and Waterman and the  $B_4$  of Reader in its stability to heat and alkali. As their paper appeared simultaneously with that of Carter, Kinnersley, and Peters (5), the question of the identity of "Factor Y" and vitamin  $B_8$  was not raised. Although the requirement of pigeons for Factor Y and that of rats for vitamin  $B_8$  have not been studied, the similarity in their stability to heat under alkaline conditions suggests the possibility that we are here dealing with one very thermostable factor which is required both by the rat and by the pigeon. Further investigation is necessary to clarify this point.

#### *"Third" Factor of Coward, Key, Morgan, and Co-workers*

In 1929 Coward, Key, and Morgan (10) reported that "light-white casein" contains a growth-promoting rat factor different from any of the

"recognized" vitamins and that it is not present in "vitamin free-casein".<sup>1</sup> Although unsuccessful in extracting this factor from yeast, they were able to obtain active alcohol and ether extracts of it from wheat embryo. The "casein" factor was destroyed by heat, at least partially. Coward, Key, Morgan, and Cambden (11) gave further evidence for the presence of a "new" growth-promoting factor in "light-white casein" (B.D.H.) and, subsequently, Coward, Key, Dyer, and Morgan (9) reported that all attempts by them to extract this growth factor from "light-white casein" had been unsuccessful. Furthermore, they demonstrated that the heating of "light-white casein" by Messrs. Glaxo in the preparation of their "vitamin-free casein" only partially destroyed this factor.

In this connection the work of Palmer and Kennedy (31) should be mentioned. They demonstrated that impure casein contains a factor other than antineuritic vitamin B that is necessary for growing rats. Later Kennedy and Palmer (26) expressed the opinion that this factor was vitamin G. There is still some possibility that they were dealing with the factor reported by Coward and co-workers. Williams and Eddy (50) also reported the presence of a thermolabile factor present in casein and brewers' yeast and necessary for the growth of rats. This factor could not be extracted from casein and in this respect and in its thermolability resembled the "casein" factor of Coward and co-workers. In 1919 Emmett and Luros (13, 14) had demonstrated that milk contains a water-soluble, heat-labile, growth-promoting vitamin other than vitamin B. Similar conclusions have been drawn by Supplee, Kahlenberg, and Flanigan (46). Whether the heat-labile factors reported by these two groups of workers will prove to be identical with that of Coward, Key, Morgan, and co-workers, future work only can determine. At present, it is impossible to judge whether the "casein" factor of Coward is identical with any of the B factors previously mentioned in this review.

#### *"Third" Factor of Hunt*

Hunt (23, 24) demonstrated the presence of a "third" factor necessary for the growth of rats and present in the residue remaining after autolyzed yeast has been extracted with weak acetic acid (0.01 per cent) and also in the fullers' earth residue obtained by treatment of the yeast extract with fullers' earth and by its subsequent extraction with concentrated barium hydroxide. Both of these residues when added to B and G fractions obtained from the same yeast gave increased growth as compared to that

<sup>1</sup> Glaxo.

obtained on the B and G fractions. Hunt suggested that the presence of the "new" factor in the fullers' earth residue might be explained by the supposition that some of the yeast residue remains in suspension in the water extract and is then adsorbed on the fullers' earth, remaining there after the extraction of the earth with barium hydroxide. He showed that the "third" factor was thermostable, as it was not destroyed when the yeast residue was autoclaved. In a subsequent paper Hunt and Wilder (25), using a stronger acetic acid (0.1 per cent) for extraction and extracting thus fourteen times, obtained the "third" factor not only in the yeast residue but in the non-adsorbable fraction after treatment with fullers' earth. Further investigation of its stability to heat demonstrated that it was stable when heated in acid medium but thermolabile in alkaline medium (pH 9). Also it was shown that the "third" factor supplements vitamin G in the prevention and cure of pellagra. The fact that the "third" factor is soluble, at least to some extent, in water and thermolabile in alkaline medium suggests its possible identity with Reader's vitamin B<sub>4</sub>. However, further work must be done to prove or disprove this hypothesis.

#### *"New" Nutritional Factors of Stiebeling*

As the result of an observed variability of symptoms of G-avitaminosis in their experimental animals on a diet supposedly deficient in vitamin G but adequate in other respects, Sherman and Sandels (44) raised the question of the possible multiple nature of vitamin G. Subsequently, and quite recently, Stiebeling (45), working in Sherman's laboratory, found that, when skim milk powder was fractionated and then fed to rats on a diet deficient only in vitamin G, better growth was obtained with the combined fractions than when double portions of either single fraction were fed. A similar observation was made when the experiment was repeated with fractions of skim milk powder prepared by a second procedure. As an explanation of these experimental findings, Stiebeling postulated the presence in the milk powder of a "new" nutritional factor necessary for rats. In support of this hypothesis, she presented a second type of evidence. When rats with an unfavorable nutritional history were given graduated portions of milk as a source of vitamin G in the Bourquin and Sherman method of assay of this vitamin, they failed to exhibit the continuous and uniform growth response throughout an eight-week period that was characteristic of the animals used in Bourquin's work. This suggested a depletion of the bodily store of some factor or factors other than those generally recognized as necessary to mammalian nutrition. Whether Stiebel-

ing was dealing with more than one "new" factor in the two types of experiments outlined above cannot be ascertained from the data presented. It is interesting to note, however, that she obtained some evidence for the presence of two other "new" factors in the vitamin B complex, but her data relative thereto were so meager that she did not include them in her report. The question of the possible identity of Stiebeling's factor or factors with any of the other so-called "third" factors of the vitamin B complex mentioned in this review cannot at this time be settled.

### *"Third Factor" of Williams and Lewis*

Our interest in the multiple nature of the vitamin B complex has extended over a period of many years. In 1921 and 1922, while working with Fay (15), we observed that the treatment of an adequate diet by different methods of heating (pressure cooking in acid and alkaline medium and baking in alkaline medium) destroyed the antineuritic factor in some cases and the rat growth factor in others, thus indicating that vitamin B was complex. In 1928 work was resumed on this problem of the multiple nature of the vitamin B complex in the hope that experimental evidence might be obtained for the presence of a factor or factors other than B and G. In 1930 Williams and Lewis (48) reported that after repeated extraction of yeast with increasing strengths of alcohol the yeast residue still retained a growth factor that supplemented the yeast extract, or the vitamin B ( $B_1$ ) and G ( $B_2$ ) fractions obtained therefrom, in producing normal growth of rats on a diet otherwise lacking in the vitamin B complex, a result that had not been obtained when the yeast extract, or its B and G fractions, constituted the sole source of B factors. We showed further that the yeast residue (Fraction R) was thermostable, and insoluble in water and alcohol. At that time we suggested the probable identity of our "third" factor and that reported by Hunt (24). Since that time work in our laboratory (Williams and Lewis, 49) has confirmed our earlier findings and demonstrated further that the yeast residue retains its potency in the "third" factor after prolonged extraction with acid alcohol, that the "third" factor is thermostable when autoclaved at 130°C for 24 hours in acid medium and when subjected to acid hydrolysis in 10 per cent hydrochloric acid at atmospheric pressure for 2 hours, that it is destroyed by heating in 10 per cent sodium hydroxide at atmospheric pressure for 2 hours, and that it is insoluble in hot water, hot alcohol, hot 10 per cent hydrochloric acid, and 50 per cent alcohol acidified to contain 1 per cent acetic acid. Still further work during the past few months (Rymer and Lewis, 43) has given addi-

tional confirmation of the presence of a third factor necessary for the normal growth of rats in the residue remaining from the aqueous-alcohol extraction of yeast, and has shown that alkaline autoclaving (5 hours; 15 lbs. pressure; pH 9.0) destroys the factor contained in the residue, whereas acid autoclaving (5 hours; 15 lbs. pressure; pH 5.3) has no effect upon it. Although our more recent results agree with those of Hunt and Wilder in showing the thermolability of our "third" factor in alkaline medium, the repeated demonstration of the insolubility of our factor in aqueous solution in contrast to the solubility of Hunt's factor makes it necessary to rescind our former view on the identity of the two factors and to reserve decision on this point for the present. The extreme insolubility of our "third" factor shows that it is not the same as the vitamin B<sub>4</sub> of Reader, although we may be dealing with a factor similar to that reported by Coward, Key, Morgan, and co-workers as present in casein. Further work must be done before the question of identity with any of the factors reported by other workers can be solved.

#### *Other Reports on "Third" Factors of the Vitamin B Complex*

In addition to the papers already mentioned, the literature contains several contributions of evidence for the existence of factors other than B and G in the vitamin B complex. The time at our disposal will not permit more than mere reference to them without any attempt to correlate the factors mentioned with the better defined factors previously reviewed. Among those that should be included are the factor necessary to prevent or cure heart block in pigeons (Carter and Drury, 3; Carter, 2); the factor whose absence leads to symptoms of intestinal stasis in rats and pigeons (Rosedale, 41, 42); the factor a deficiency of which causes muscular dystrophy in the guinea pig and the rabbit but not in the rat (Goetsch and Pappenheimer, 17); and the factor which possesses the power of counteracting the ill effects resulting from the ingestion of dried egg white by rats (Boas, 1; Fixsen, 16).

Guha and Drummond (19) have presented evidence which points to the multiple nature of vitamin B. The existence of two factors other than vitamins B and G, one in yeast and the other in milk, both of which are required for the normal growth of rats, has been claimed by Guha (18). Sure, Smith, and Kik (47) have suggested that vitamin G is composed of two components, the anti-dermatitis factor for which the G should be retained, and a growth factor for which they suggest the term vitamin F. It has been shown by Hartwell (22) that the factor which prevents kidney



abnormalities when high protein diets are fed to young rats is not the antineuritic vitamin B. Whether vitamin G or some other factor in the B complex is the "protective" agent is not shown by her work. This question has been under investigation in our laboratory (Race, Longwell, and Lewis, 33) recently.

### *Correlation of "Third" Factors*

Thus, we see that our present knowledge concerning factors other than B and G in the vitamin B complex is in an extremely chaotic state. It seems hardly possible that there are as many factors as have been suggested; and yet the evidence at hand makes it impossible to do otherwise than to conclude that the vitamin B complex is composed of several definite entities and some that are less well defined. However, it seems altogether probable that further investigation will prove that some of the alleged factors are identical and that, instead of having as many components as are now claimed, the number will be reduced.

For the determination of the possible identity of certain so-called third factors of the vitamin B complex, two chief criteria have been used, namely their comparative requirements by the pigeon and by the rat and their relative thermostability. Unfortunately, the data in the literature on the latter point leave much to be desired, as there has been no uniformity in the conditions used by different investigators for the determination of the

TABLE I  
"THIRD" FACTORS OF THE VITAMIN B COMPLEX

Name or tentative designation	First reported by	Required by		Behavior to alkali and heat
		Adult pigeon	Growing rat	
Vitamin B <sub>3</sub>	Williams and Waterman	Yes	No	Labile
Vitamin B <sub>4</sub>	Reader	No	Yes	Labile
Vitamin B <sub>5</sub> *	Peters et al.	Yes	(?)	Very stable
Factor Y*	Chick et al.	(?)	Yes	Very stable
"Third" Factor	Coward et al.	(?)	Yes	Labile
"Third" Factor	Hunt	(?)	Yes	Labile†
"New" Nutritional Factor	Stiebeling	(?)	Yes	(?)
"Third" Factor	Williams and Lewis	(?)	Yes	Labile†

\* Possible identity of these two factors suggested in this review.

† At first reported to be thermostable.

behavior of the different factors to heat. The importance of pH control in such comparative studies has been emphasized by Williams, Waterman, and Gurin (53), and by Chick and Copping (6). Certainly, unless the effect on two factors is studied under identical conditions of hydrogen ion concentration, temperature and degree of moisture, accurate conclusions as to the relative stability of the two factors cannot be made. Failure to observe these precautions has led to many contradictory statements in the literature as to the stability of the different factors of the vitamin B complex. However, since much of the evidence for the identity, or lack thereof, of the various so-called "third" factors has been based upon their stability or instability to heat, this information and an indication of whether the particular factor is required by the pigeon and by the rat are given in Table I.

#### CONCLUSION

In conclusion, we may well ask ourselves several questions. Which of these components of the vitamin B complex are required by man? Does he need all of these alleged factors claimed as vital for normal nutrition of the rat and of the pigeon? What deficiency diseases other than beriberi and pellagra may be a result of, or complicated by, a lack of these "newer" components of the vitamin B complex in the diet? Are beriberi and pellagra simple deficiency diseases due to an inadequate supply of a single specific vitamin in each case, or is a lack of two or more components of the vitamin B complex the basis of their etiology? Although the last question, particularly as regards pellagra, is receiving considerable attention, much additional information must be gathered before our knowledge concerning the complete rôle of the multiple components of the vitamin B complex in the maintenance of health and normal nutrition will be a closed chapter.

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